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Thesis Title:

Expression Of CD8 On Mouse Alveolar Macrophages

by

Geneviève Isabelle Nault

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Master of Science**.

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Expression Of CD8 On Mouse Alveolar Macrophages** submitted by **Geneviève Isabelle Nault** in partial fulfillment of the requirements for the degree of **Master of Science in Experimental Medicine**.

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ABSTRACT

Rat alveolar macrophages (AM) and mast cells express a CD8 molecule that differs from the one on T cells, because it is not recognized by antibodies that binds to the immunoglobulin-like region of CD8α on T-cells. Thus, we hypothesized that mouse AM also express CD8. Expression of CD3 and CD8 mRNA in bronchoalveolar lavage (BAL) cells from Balb/c and C57Bl/6 mice was investigated by reverse-transcriptase polymerase chain reaction (RT-PCR). Samples positive for CD8 but negative for CD3 were identified, suggesting that CD8 expressing cells are not T- cells. Single-cell RT-PCR was also used to study CD8 and CD3 expression in 16 different BAL cells with morphological characteristics of macrophages. Four cells expressed CD8 and not CD3, confirming the existence of a non-T-cell population expressing CD8 in mouse lungs. Protein expression of CD8 could not be detected by flow cytometry or western blot. These results suggest that a subpopulation of mouse AM expressed CD8.

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TABLE OF CONTENTS

Chapter		Page
1	**INTRODUCTION, RATIONALE, AND HYPOTHESIS**	1
	I. INTRODUCTION	2
	II. BACKGROUND	2
	A. CD8 molecule	2
	1) Structure	2
	2) Ligands	6
	3) Expression of CD8 on cytotoxic T lymphocytes	7
	4) Functions of CD8 on T lymphocytes	8
	5) Expression of CD8 on other immune cells	10
	• CD8 expression on other lymphocyte subsets	10
	• CD8 expression on dendritic cells	11
	• CD8 expression on mast cells	11
	• CD8 expression on monocytes/macrophages	12
	6) CD8 functions on cells other than T cells	14
	• CD8 function on other lymphocyte subsets	14
	• CD8 function on dendritic cells	14
	• CD8 function on mast cells	15
	• CD8 function on monocytes/macrophages	15
	B. Macrophages	16
	1) Development	16
	2) Macrophage subtypes in the lung	16
	3) Alveolar macrophage functions	17

III. SUMMARY 19

IV. RATIONALE 19

V. HYPOTHESIS 20

VI. OBJECTIVES 20

2 **MATERIALS AND METHODS** 21

I. REAGENTS 22

II BUFFERS 23

III. ANIMALS 24

IV. ISOLATION OF CELLS FROM LUNGS AND FROM SPLEEN 25

V. CELL LINES 26

VI. PRIMER DESIGN 27

VII. RNA EXTRACTION 28

VIII. REVERSE-TRANSCRIPTASE AND POLYMERASE CHAIN REACTION 29

IX. CLONING OF PCR PRODUCTS 33

X. SINGLE-CELL RT-PCR PROCEDURE 34

XI. FLOW CYTOMETRY 35

XII. WESTERN BLOT 36

- Protein isolation 36
- Electrophoresis/Transfer/Detection 36

XIII. MONOCLONAL ANTIBODY PRODUCTION 40

- Peptide design 40
- Immunization 40
- Selection and cloning of hybridomas 41
- Enzyme-linked immunosorbent assays (ELISA) 41

XIV. STATISTICS 42

3 **RESULTS** 43

I. Bronchoalveolar lavage 44

II. Analysis of CD8 expression in Balb/c mice BAL cells 45

A. PCR analysis for CD8α and β in splenic and BAL cells 45

B. Comparison of CD3 and CD8 expression in BAL cells 50

C. Single-cell RT-PCR analysis 52

III. PCR analysis for CD8 expression in BAL cells from C57Bl/6 mice 59

IV. PCR analysis for CD8 expression in murine macrophage cell lines 61

V. Flow cytometric analysis 61

A. Rat anti-mouse CD8 antibody 61

B. Mouse anti-rat CD8 antibody 62

VI. Western blot analysis 69

A. Rat anti-mouse CD8 antibody 69

B. Mouse anti-rat CD8 antibody 69

C. D9, a monoclonal mouse anti-mouse, rat and human CD8α antibody 74

D. H-160, a polyclonal rabbit anti-mouse, rat and human CD8α antibody 77

4 **DISCUSSION AND FUTURE DIRECTIONS** 80

I. DISCUSSION 81

A. Bronchoalveolar lavage 81

B. mRNA expression 81

C. Protein expression 87

D. Expression of CD8 on multiple cell types 89

E. Proposed roles of CD8 on cells other than T cells 89

F. Differential marker expression between rat and mouse 91

G. Conclusions 92

II. FUTURE DIRECTIONS 93

A. To expand the findings provided by single-cell RT-PCR 93

B. To find an Ab that recognizes CD8 on mouse AM by flow cytometry 93

C. To extend our studies to other cell types in the mouse 94

D. To pursue the study of CD8 in C57Bl/6 mice 94

INDEX OF TABLES

Chapter	Table	Title	Page
1	1	CD8 molecular weight and glycosylation sites	5
	2	Expression of CD8 on multiple cell types	7
2	3	Primer sequences and product size	30
	4	Antibodies	38-39
3	5	Total cell numbers obtained in BAL samples	44
	6	Differential cell counts for BAL samples	45
	7	CD8 and CD3 mRNA expression in different BAL samples	52
	8	Single-cell PCR on BAL cells: CD8 and CD3 expression	59

INDEX OF FIGURES

Chapter	Figure	Title	Page
1	1	Classical interaction of CD8 and TCR with MHC I	3
2	2	Mapping of CD8 primer pairs	31-32
3	3	CD8α and CD8β mRNA expression in Balb/c splenic cells	46
	4	Sequence analysis of product 3α from splenic cells	47
	5	CD8α and CD8β mRNA expression in Balb/c BAL cells	48
	6	Sequence analysis of product 5α from BAL cells	49
	7	CD3 and CD8 expression in different BAL samples	51
	8	Single-cell PCR: Experiment #1	53
	9	Single-cell PCR: Experiment #2 β-actin expression in BW5147 cells and macrophages	54
	10	Single-cell PCR: Experiment #2 CD3 and CD8 expression in BW5147 cells	55
	11	Single-cell PCR: Experiment #2 CD3 and CD8 expression in macrophages	56
	12	Single-cell PCR: Experiment #3 Controls and β-actin expression in macrophages	57
	13	Single-cell PCR: Experiment #3 CD3 and CD8 expression in macrophages	58

14	β-actin, CD3, CD8α and CD8β mRNA expression in C57Bl/6 BAL samples	60
15	β-actin, CD3, CD8α and CD8β mRNA expression in macrophage cell lines	63
16	CD8α protein expression on mouse splenic cells using 53-6.7 antibody	64
17	CD8β protein expression on mouse splenic cells using 53-5.8.3 antibody	65
18	CD8α protein expression on mouse alveolar macrophages using 53-6.7 antibody	66
19	CD8β protein expression on mouse alveolar macrophages using 53-5.8.3 antibody	67
20	CD8α protein expression on mouse splenic cells using OX-8 and 341 antibodies	68
21	Western blot analysis using 53-6.7 antibody	71
22	Western blot analysis using OX-8 antibody	72
23	Western blot analysis using OX-8 antibody after urea treatment	73
24	Western blot analysis using D9 antibody	75
25	Western blot analysis using D9 antibody after urea treatment	76
26	Western blot analysis using H-160 antibody	78
27	Western blot analysis using H-160 antibody after urea treatment	79

ABBREVIATIONS

Ab	Antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity
Ag	Antigen
AIDS	Acquired immunodeficiency syndrome
AM	Alveolar macrophage
APC	Antigen presenting cell
APS	Ammonium persulfate
BAL	Bronchoalveolar lavage
BCIP	5-bromo-4-chloro-3-indoyl-phosphate
BLAST	Basic Local Alignment Search Tool
βME	β-mercaptoethanol
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CFA	Complete Freund's adjuvant
ConA	Concanavalin A
d	Day
DC	Dendritic cells
DMEM	Dulbecco's Modified Eagle's Medium
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EtBr	Ethidium bromide

FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
h	Hour
HEPES	N-(2-hydroxyethyl) piperazine-N (2-ethanosulfonic acid)
HLA	Human Histocompatibility Leukocyte antigen
HSLAS	Health Sciences Laboratory Animal Services
ICFA	Incomplete Freund's adjuvant
Ig	Immunoglobulin
IL	Interleukin
IM	Interstitial macrophage
IPTG	Isopropyl-β-D-thiogalactoside
KLH	Keyhole limpet hemacyanin
LB	Luria Bertani
LCMV	Lymphocytic Choriomeningitis Virus
mAb	Monoclonal antibody
MC	Mast cell
MHC	Major histocompatibility complex
min	Minute
mRNA	Messenger ribonucleic acid
NCBI	National Center of Biotechnology Information
NK	Natural killer
NO	Nitric oxide
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PG	Prostaglandin
PHA	Phytohemagglutinin
PM	Peritoneal macrophage
PMC	Peritoneal Mast Cell
PVDF	Polyvinylidene difluoride
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	reverse transcriptase
s	Second
sCD8	Soluble CD8
SDS	Sodium dodecyl sulfate
SLE	Systemic lupus erythematosus
TBS	Tris buffered saline
TCR	T cell receptor
TEMED	Tetramethylethylenediamine
TGF-β1	Transforming growth factor-β1
Th	T helper lymphocyte
TL Ag	Thymic Leukemia Ag
TNF	Tumor necrosis factor
wk	Week
X-Gal	5-bromo-4chloro-3-indolyl-β-D-galactoside

CHAPTER 1

INTRODUCTION, RATIONALE AND HYPOTHESIS

I. INTRODUCTION

The expression of CD4 or CD8 molecules was initially believed to correlate with lymphocytes exhibiting distinct effector functions, helper T cells $CD4^+$ and killer T cells $CD8^+$ (1). It was later observed that some $CD4^+$ cells can be cytotoxic (2) and some CD8 cells can produce many cytokines (3). The role of CD4 and CD8 was then correlated with T cell specificity in its MHC restriction rather than with T cell function (4). Subsequently, the CD8 molecule was identified on cell types other than T lymphocytes and thus it may have wide-reaching roles in the regulation of immune cell functions.

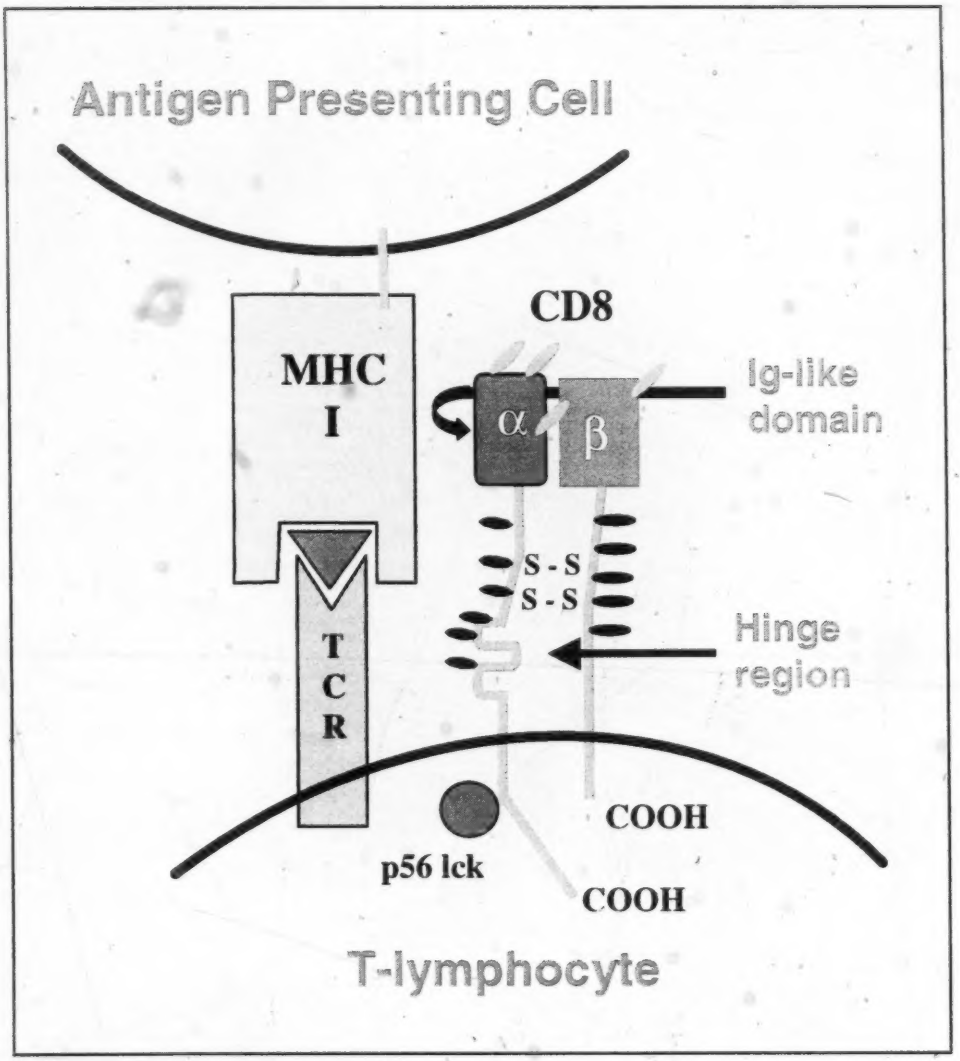
In previous studies our lab identified an atypical CD8 molecule on rat alveolar and peritoneal macrophages (5). This CD8 molecule appears to have a novel structure and the potential to regulate macrophage functions. To gain further insight into the expression pattern of CD8 on non-T cells, we chose to investigate the presence of CD8 on alveolar macrophages (AM) in mice.

II. BACKGROUND

A. CD8 molecule

1) Structure

The CD8 molecule is composed of disulfide-linked α and β subunits. It is predominantly expressed on cytotoxic T lymphocytes and is found as a αβ heterodimer or a αα homodimer (6,7). Recent evidence suggests that human CD8β chains can be expressed as a homodimer on transfected cells (8).



N-linked glycosylation

O-linked glycosylation

Figure 1: Classical interaction of CD8 and T cell receptor (TCR) with Major Histocompatibility Complex I (MHC I).

However, in species other than humans, expression of CD8β on the cell surface depends on the co-expression of CD8α (9,10).

The tertiary structures of the α and β chains are similar. Both chains consist of an NH_2-terminal domain homologous to immunoglobulin (Ig)-like variable domains, a hinge or "linker" region that is 20 amino acids shorter in the β chain (11), a membrane-spanning region and a cytoplasmic tail domain (Figure 1). The Ig-like domain is responsible for the interaction between CD8 and the major histocompatibility complex class I (MHC I) molecule composed of one chain containing 3 domains (α1, α2 and α3 (the most conserved domain)) and β2-microglobulin allowing stable expression of MHC I. The α chain is more efficient than the β chain in mediating the interaction between CD8 and MHC I (12). The hinge region provides flexibility to the CD8 molecule that facilitates its interaction with MHC I. The cytoplasmic tail of the α chain is longer than the tail of the β chain and includes a binding site for a src-family tyrosine kinase, $p56^{lck}$ (13).

Mouse CD8α and CD8β have molecular weights of 38 kDa and 30 kDa respectively (14,15) (Table 1). The mouse α chain has three N-linked glycosylation sites, while the β chain has one site (10,16). In rats, CD8α is 32 kDa and CD8β is 37 kDa (17,18). Although 78% of the mouse CD8β residues are identical to rat CD8β, the rat β chain contains three N-linked glycosylation sites, which could account for its larger size in comparison to mouse CD8β (6,10). Human CD8α and β are 32 and 34 kDa respectively (15,19,20). Human

CD8α has no N-linked glycosylation sites, whereas CD8β has one N-linked glycosylation site (19,21). All species have O-linked glycosylation sites, mostly located within the hinge region (11,22).

Table 1: CD8 Molecular Weight And Glycosylation Sites

	Molecular weight		**N-glycosylation sites**	
Species	CD8 α	CD8 β	CD8 α	CD8 β
Human	32 kDa	34 kDa	0	1
Rat	32 kDa	37 kDa	1	3
Mouse	38 kDa	32 kDa	3	1

In addition to interspecies differences in CD8 protein, there are also differences in gene transcription. Transcription of mouse CD8α gene produces two mRNAs, one full length CD8α and one alternatively spliced CD8α' in which a 31 base pair deletion leads to a truncated cytoplasmic tail in the protein (23). The protein resulting from the α' mRNA is expressed mainly on thymocytes and not on peripheral T lymphocytes (24). Human CD8α also has an alternative mRNA which results from splicing of CD8α affectively removing the transmembrane region, resulting in a soluble form of CD8 (25,26). Soluble CD8 has been detected in various disease states such as acquired immunodeficiency syndrome (AIDS) or systemic lupus erythematosus (SLE) (27-30). Human CD8β mRNA also has splice variants affecting the transmembrane domain and

cytoplasmic tail, but no studies have described their translation into functional proteins (21,25).

2) Ligands

MHC I is the classical ligand for CD8. T lymphocyte CD8 binds to the α2 and α3 domain of MHC I expressed by an antigen presenting cell (APC) or target cell. CD8 binding to MHC I creates a stable interaction between the T cell receptor (TCR) and the peptide/MHC I complex.

Novel ligands for CD8 have recently been identified. A glycoprotein, gp180, expressed on intestinal epithelium has the ability to activate $CD8^+$ T lymphocytes and this stimulation is inhibited by an Ab to CD8, but is not affected by an Ab to MHC class I (31,32). A protein referred to as the "T lymphocyte triggering factor" derived from *Trypanosoma brucei* also binds CD8 and induces interferon-γ (IFN-γ) release from $CD8^+$ lymphocytes (33).

Non-classical MHC molecules also bind CD8. In mice, thymic leukemia (TL) Ag is expressed on the intestinal epithelium and interacts with CD8 (34). When binding TL Ag, CD8 interacts exclusively with the α3 domain. Human histocompatibility leukocyte Ag-G (HLA-G) also binds CD8. Binding of CD8 to placental cytotrophoblasts which express HLA-G, but none of the classical HLA molecules, has been described (35). This latter observation is surprising because the HLA-G α3 domain has a substitution at a position thought to be critical for CD8 binding.

3) Expression of CD8 on cytotoxic T lymphocytes

CD8 was first described in T lymphocytes and is the receptor that differentiates cytotoxic T cells ($CD8^+$) from helper T cells ($CD4^+$). CD8 is expressed as an α/β heterodimer on thymus-derived T cells and as an α/α homodimer on thymus-independent T lymphocytes found in selected sites such as intestinal epithelium (36-38). During the past 10 years, a number of reports have described CD8 expression on various cells of the immune system (Table 2).

Table 2: Expression of CD8 on multiple cell types

Cell types	CD8 α	CD8 β	mRNA/Protein	References
Rat Macrophages				
Alveolar	+	+	mRNA + protein	(5,39,40)
Peritoneal	+	+	mRNA + protein	(5)
Microglia	+	+	protein	(41)
Pre-Kupffer	+	-	mRNA + protein	(42)
Human macrophages				
Alveolar*	+	NT	protein	unpublished
Monocytes**	+	NT	protein	unpublished
NK cells				
Human	+	-	protein	(43,44)
Rat	+	-	protein	(45,46)
Mouse	-	-	protein	(47-49)

Dendritic cells				
Human thymic cells	-	-	protein	(50)
Rat thymic cells	+	NT	protein	(51)
Mouse thymic cells	+	+	mRNA + protein	(52-54)
Mouse splenic cells	+	-	mRNA + protein	(55)
Mast cells				
Human	NT	NT		
Rat	+	+	mRNA + protein	(40)
Mouse cell line	+	-	cDNA/protein	(56)
$CD4^+$ T lymphocytes†	+	NT	mRNA + protein	(29,57-59)
B lymphocytes‡	+	NT	mRNA + protein	(60-62)

NT Not tested
* Samples from Sarcoidoisis and Small cell cancer patients.
** Healthy donors and Dengue fever patients.
† ConA and IL-4 activated lymphocytes.
‡ Human B cell lymphocytic leukemia.

4) Functions of CD8 on T lymphocytes

CD8 can act as an adhesion molecule by binding to MHC I to enhance the affinity of T-cells for their target cells (63). The binding sites for CD8 on MHC I are numerous, suggesting that CD8 could interact in different ways with MHC molecules. It has even been described that the empty binding groove of MHC I can bind to mature CD8 protein or peptides derived from the amino terminus of both CD8α and CD8β (64). The α2 and α3 domains of MHC I are involved in the interaction between CD8 and MHC I (65-67). The β_2-microglobulin subunit of

MHC I is also important for its interaction with mouse CD8 (34). CD8 also acts as a co-signaling protein with the TCR-CD3 complex (68). The Ig-like region of the CD8α chain and the TCR must bind the same MHC I molecule to deliver maximal activation signals to the cell (68).

Cells of mice lacking CD8α chain cannot express CD8β on their surface (9,10). Mice homozygous for a CD8α deficiency are healthy and fertile (69) and compared to wild type mice, have similar numbers of cells in lymphoid organs. However, CD8 deficiency results in the absence of functional cytotoxic T cells as indicated by studies using lymphocytic choriomeningitis virus (LCMV) infected cells as targets (69,70). Studies of CD8 knockout mice have shown that CD8 is required for positive selection in the thymus. The role of CD8 in negative selection in the thymus seems to be dependent on the Ag involved (71).

To study CD8-mediated cell signaling, mice deficient for the cytoplasmic tail of CD8α were generated. These mice had a decrease in the number of $CD8^+$ T cells which demonstrated cytotoxic effects suggesting that CD8 signaling is required for maturation but not for delivering cytotoxic signals to target cells (69).

There is reduced number of $CD8^+$ cells in CD8β knockout mice. The CD8β chain is not essential for the cytotoxic effector functions of $CD8^+$ T-lymphocytes (72). However, in the presence of a CD8 αβ heterodimer, CD8β increases CD8 avidity for TCR-ligand complex (73). Moreover, the β chain enhances the association of CD8α with the tyrosine kinase $p56^{lck}$ (73) and avidity of TCR for its ligand (74).

5) Expression of CD8 on other immune cells

- *CD8 expression on other lymphocyte subsets* (Table 2)

Natural Killer (NK) cells are large granulated lymphocytes and are cytotoxic to altered cells such as tumor cells. NK cells express CD16, a receptor that binds the Fc portion of IgG, and are capable of antibody-dependent cell-mediated cytotoxicity (ADCC) (49). Human and rat NK cells express a homodimer of CD8α but not the CD8β chain (43-46). Expression of CD8 has not been detected in mouse NK cells (47-49).

The helper T lymphocyte (Th) subset is characterized by expression of CD4. Th have under some circumstances, been reported to co-express CD8α and CD4. Concanavalin A (ConA)-activated Th cells treated with a glucocorticoid, dexamethasone, upregulated CD8 expression. (57) Rheumatoid arthritis (RA) patients also express CD8 on $CD4^+$ T lymphocytes (29). CD8α upregulation on CD4+ T cells seems to be related to the degree of cell activation, because CD8 expression is induced on rat and human CD4+ T cells treated with IL-2 and ConA (57,58) or phytohemagglutinin (PHA) (58,59).

B lymphocytes are involved in humoral immunity and express CD8 in certain disease states. About 7% of $CD19^+$ B lymphocytes from individuals with AIDS express CD8α (60). As many as 68% of $CD19^+$ B lymphocytes from patients with chronic lymphocytic leukemia were $CD8\alpha^+$ (61,62)

- *CD8 expression on dendritic cells* (Table 2)

Dendritic cells (DC) are the major APC of the immune system. Mature DC constitutively express accessory molecules necessary for lymphocyte activation. After Ag uptake, DC migrate to the draining lymph node to present processed Ag to lymphocytes, thus initiating an immune response (75,76).

Different populations of DC have been described and CD8 is one of the markers important in the classification of these DC populations. The expression of CD8 on mouse splenic DC depends on the location of the cells. A majority of $CD8\alpha^-$ DC reside at the margin between the red and the white pulp, whereas most $CD8\alpha^+$ DC are present in periarteriolar lymphocytic sheaths rich in T cells (55). Mouse thymic DC express mRNA and protein for both CD8α and β (52-54). The level of expression for CD8β in mouse thymic DC is low and CD8β is undetectable on splenic DC (53,77).

In rats, 34% of thymic DC cells expressed CD8α (51). It is important to note that human thymic DC appear to be $CD8^-$, but express CD4 instead (50).

- *CD8 expression on mast cells* (Table 2)

Mast cells (MC) are a key player in allergies. Mediators contained inside their granules are released upon activation. One of the major mediators is histamine, which is responsible for many of the symptoms seen during allergic reactions. MC also release proteases and a variety of cytokines such as tumor necrosis factor (TNF) (78,79).

Expression of CD8α mRNA and protein has been described on the mouse MC line MC9. The cells contain very few granules and might not be representative of *in vivo* MC (56). More recently, however, flow cytometric analysis has shown that 68% of rat peritoneal mast cells (PMC) express CD8α and 28% express CD8β (40). These cells also express mRNA for CD8α and β. To date, there are no studies describing CD8 expression on human MC.

The CD8 molecule expressed by rat MC is unusual because an antibody (Ab) to the Ig-like domain of CD8α (G28) did not detect CD8 on PMC, although an Ab to the hinge region of CD8α (OX8) detected CD8α on these cells (40). So far, the difference between T cell CD8 and MC CD8 is unclear, but it seems to involve post-translational modifications since the mRNA sequence of MC CD8 is identical to the one described in T cells.

- *CD8 expression on monocytes / macrophages* (Table 2)

Descriptions of CD8 expression on macrophages come mainly from animal models of injury. The identification of CD8 on monocyte/macrophage was first reported in a rat model of combined injury which was a whole-body irradiation followed by a thermal injury (80). Seven days after CI, 40% of peripheral blood mononuclear cells were $CD8^+$ and were identified as monocytes because they lacked a lymphocyte marker CD5. Studies of acute allograft rejection (81) and axonal degeneration in the peripheral nervous system (82) observed recruitment of $CD8^+$ $CD4^+$ monocytes to the injury site.

Additional evidence for CD8 expression by monocytes was described in a model of focal ischemia in the rat brain (41). A change in cell phenotype was observed at the injury site and showed a decrease in $CD4^+$ macrophage/microglia and an increase in $ED1^+$ (marker for monocytes) $CD8^+$ cells after 3 d. Few $CD8^+$ cells were detected 14 d after injury. Several other injury models have described the infiltration of $CD8^+$ cells with features of macrophages at the injury site (83,84).

Expression of CD8 on cells of the macrophage lineage has also been observed under normal conditions. For example, 90% of pre-Kupffer cells in the rat liver express CD4 and 73% express CD8α but not CD8β (42). These cells were negative for other T lymphocyte surface markers. Expression of CD8 was lost during maturation of the pre-Kupffer cells into Kupffer cells with only 7% of mature cells still expressing CD8.

Rat alveolar and peritoneal macrophages (PM) express protein for both CD8α and CD8β (5). Expression of CD8 was detected on 63% of the cells for the α chain and 52% for the β chain on AM, whereas 49% and 37 % of PM expressed CD8 α and β, respectively. These cells were also positive for CD8α mRNA (39). As described for CD8 on MC, the molecule expressed by AM and PM differs from CD8 on T cells.

Human macrophages and monocytes also express CD8. Flow cytometry analysis showed CD8α expression on the human cell line THP-1 (85) and CD8 was also detected on monocytes during and 9 months after dengue fever (86).

6) CD8 functions on cells other than T cells

Few studies have explored the role of CD8 on cell types other than lymphocytes. The proposed functions are controversial and require further study.

- *CD8 function on other lymphocyte subsets*

Expression of CD8 on $CD4^+$ lymphocytes and B cells has been described in various diseases and is accompanied by the presence of a soluble form of CD8 (sCD8) in the serum (27,29,30). A possible role suggested for CD8 is as part of a defense mechanism against the high numbers of activated inflammatory cells found in patients with RA or SLE (29,30). It has been suggested that CD8 could be secreted to bind to MHC class I on activated cells and suppress their function or induce apoptosis (87,88). However, in one case of a patient with chronic lymphocytic leukemia, an increased in $CD8^+$ B lymphocytes correlated with accelerated disease progression (89).

- *CD8 function on dendritic cells*

Most studies have described CD8 function on DC as immunosuppressive. Studies comparing $CD8^-$ DC to $CD8^+$ DC for their ability to stimulate T cells show that DC expressing CD8 were poor inducers of T cells proliferation compared to $CD8^-$ DC (90). They also observed that $CD8^+$ DC had suppressive effects during direct contact with lymphocytes. Analysis of the factors that determined whether APC induce downregulation or death of interacting T cells led to the conclusion that CD8 was a crucial molecule on APC, and that CD8

could transmit negative signals into the T cell through interaction with MHC I (87,88). Other studies failed to support this theory. DC from $CD8^{-/-}$ mice were sorted according to their expression of DEC 205 which correspond to the expression of CD8 in normal mice since $CD8^+$ DC are also DEC 205^+ and $CD8^-$ DC are DEC 205^-. Results were similar using $CD8^+$ DC from from normal mice and DEC 205^+ DC from $CD8^{-/-}$ mice, suggesting that CD8 is not required for the regulatory function of $CD8^+$ DC (91).

- *CD8 function on mast cells*

Interesting results have been obtained from cross-linking studies of CD8 on rat MC. OX8, an Ab recognizing the hinge region of rat CD8α was used to cross-link CD8. TNF and nitric oxide (NO) but not histamine were released by MC following CD8 cross-linking, suggesting that CD8-induce mediator release is selective (92). The identification of a ligand for the novel CD8 molecule expressed by MC will be a valuable step in the elucidation of CD8 functions on MC.

- *CD8 function on macrophages/monocytes*

OX-8 Ab has also been used to cross-link CD8 on rat AM. Stimulation of CD8 led to the release of TNF and IL-1β from AM (93). In addition, OX-8-mediated cross-linking of CD8 on rat AM produced NO and induce AM to kill *Leishmania major* (94).

It remains to be demonstrated whether MHC I is a ligand for macrophage CD8. As the ligand binding portion of the macrophage CD8α varies from CD8 on T cells (negative for G28 epitope on macrophages) (5), it is possible that it has different binding characteristics and binds alternative ligands. The identification of the appropriate ligand for CD8 on various cell types will provide better insight into the functions of CD8.

B. Macrophages

1) Development

Macrophages originate from bone marrow, where their earliest progenitor is the myelomonocytic stem cell (95). This stem cell proliferates and differentiates in the bone marrow into a monocyte before entering the circulation. From the blood, monocytes migrate to a variety of tissues where they further differentiate into macrophages. The macrophage life-span varies from months to years depending on its activities and location. Macrophage maturation is tissue dependent and leads to the appearance of different macrophage populations such as microglial cells in the nervous system, Kupffer cells in the liver and AM in the lungs.

2) Macrophage subtypes in the lung

There are several types of macrophages within the lung including AM, interstitial tissue macrophages (IM), intravascular macrophages, and DC, which share certain phenotypic characteristics with macrophages. AM are the most

abundant inflammatory cell in the airways and represent over 90% of the cells harvested by bronchoalveolar lavage (BAL) from normal lungs. IM are located in the connective tissue of the lungs and are smaller than AM (96). They are capable of phagocytosis but might be more important in antigen presentation because they express high levels of MHC II molecules on their surface (96,97). Intravascular macrophages have been described in many species (98,99) and are closely associated with the endothelium of pulmonary capillaries (99). They are highly phagocytic and are important for the removal of particles and bacteria from the blood (98,100). DC are also found in the interstitial tissue in small numbers. They have little phagocytic function but express high levels of MHC II molecules important for their accessory function in the activation of T lymphocytes (97,101). Very few cells with characteristics of IM or DC are found in BAL fluid (102,103).

Under normal circumstances and during inflammation, AM are replenished from (1) bone marrow-derived blood monocytes that migrate into the alveoli (104-107), (2) migration of IM into the lung lumen (108,109), and (3) local proliferation of AM (105,110). However, AM have limited proliferative potential.

3) AM functions

AM maintain the first line of defense against inhaled foreign Ag and pathogenic microorganisms. They are the only macrophage population in the lung in direct contact with the external environment. AM are efficient at removing inhaled particles through phagocytosis and they have important microbicidal

actions. These essential functions are exerted via the release of over 100 immunological and inflammatory mediators by macrophages (100,111). The mediators include oxygen radicals, NO, proteases, cytokines, chemokines and growth factors.

AM also down-regulate pulmonary immune responses, thus limiting damage to the lungs. This immunosuppressive effect of macrophages was demonstrated by elimination of AM through instillation of liposome containing dichloromethylene diphosphonate into the lungs. Depletion of AM amplified the response to inhaled allergen by increasing migration of mononuclear cells into the lungs, potentiating the antigen presentation capacity of DC and increasing the clonal expansion of T lymphocytes (112).

A variety of mediators are involved in the suppressive effects of AM. The secretion of transforming growth factor-β1 (TGF-β1) and prostaglandins (PG) suppresses lymphocytic responses to interleukin-2 (IL-2), an activator of lymphocyte proliferation (113,114). Moreover, the release of NO and TNF inhibits the maturation and antigen presenting ability of DC and, therefore, the inductive signals to naive T-cells (115). AM acquire their suppressive effect as they mature (116).

AM can also promote immune responses. They have a role in T cell proliferation, as it has been demonstrated that the production of IL-1β by AM is essential for primary immune responses to T cell antigens. Blocking IL-1 secretion by macrophages abrogates the immune response (117,118). Moreover,

AM stimulated with Ag have been shown to recruit T cells, specific for that Ag, into the lungs (119).

III. SUMMARY

It is clear that CD8 expression is not limited to T cells. Moreover, there is heterogeneity in the form of CD8 expressed in different cell subsets, species and disease states. Given the important role of CD8 on T cells and promising evidence that CD8 on macrophages plays a role in activation of rat macrophages, more studies are required to elucidate the function and structure of CD8 on cell types other than lymphocytes.

IV. RATIONALE

The observation that cross-linking of CD8 on macrophages led to mediator release suggested that CD8 can be a regulatory receptor for macrophage functions. Accordingly, we wanted to elucidate the nature of macrophage CD8 and the molecular mechanisms and signaling pathways activated following ligation of this CD8. Unfortunately, studies of CD8 in rats were limited by the availability of reagents. Therefore, we proposed to study CD8 expression by mouse AM.

A mouse model could present advantages over the rat because: 1) the structure and function of CD8 on T cells has been more extensively characterized in mice; 2) the range of reagents to study signaling pathways is greater, and 3) the

existence of knockout mice provides a novel tool to study CD8 function on macrophages.

V. HYPOTHESIS

Mouse alveolar macrophages express CD8.

VI. OBJECTIVES

1. To determine if mouse alveolar macrophages express mRNA for CD8.
2. To determine if CD8 protein is expressed by mouse alveolar macrophages.

CHAPTER 2

MATERIALS AND METHODS

I. REAGENTS

The following reagents have been purchased from Invitrogen, Burlington, Ontario: Dulbecco's modified Eagle's medium (DMEM) (high glucose and regular), TRIzol reagent, Oligo(dT), Maloney's Murine Leukemia Virus (M-MLV) reverse transcriptase enzyme, 5X first strand buffer, dNTP's, Magnesium chloride($MgCl_2$), Taq DNA polymerase, 2 Kb bp ladder, Invitrogen's T/A Cloning® kit, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), Luria Bertani (LB) broth, RNAse inhibitor, Superscript II, Bench Mark molecular weight protein markers, urea.

The following reagents have been purchased from Sigma, Toronto, Ontario: ethylenediaminetetraacetic acid (EDTA), RPMI 1640, L-glutamine, penicillin/streptomycin, sodium pyruvate, sodium bicarbonate ($NaHCO_3$), β-mercaptoethanol (βME), N-(2-hydroxyethyl) piperazine-N (2-ethanosulfonic acid) (HEPES), sodium chloride (NaCl), glucose, RNAse treated water, isopropyl-β-D-thiogalactoside (IPTG), ampicillin, Genelute™ Plasmid Miniprep kit, di-sodium hydrogen orthophosphate (Na_2HPO_4), Tris base.

The following reagents have been purchased from BioRad, Mississauga, Ontario: DC protein assay kit, dithiothreitol (DTT), 40% acrylamide (29:1), ammonium persulfate (APS).

The following reagents have been purchased from Fisher Scientific, Nepean, Ontario: tetramethylethylenediamine (TEMED), isopropanol, chloroform, potassium chloride (KCl), Tween 20, sodium dodecyl sulfate (SDS), glycine, 12 N hydrochloric acid (HCl).

The following reagents have been purchased from Amersham Biosciences, Baie d'Urfé, Quebec: polyvinylidene difluoride (PVDF) membrane, chemiluminescence detection kit.

The following reagents have been purchased from EMD Chemicals Inc., Gibbstown, NJ: potassium di-hydrogen orthophosphate (KH_2PO_4), calcium chloride dihydrate ($CaCl_2$-2 H_2O), Magnesium chloride hexahydrate ($MgCl_2$-6 H_2O).

SDS loading buffer has been purchased from New England BioLabs Inc, Pickering, Ontario, Rompun from Bayer, Toronto, Ontario, Ketalean from MTC Pharmaceuticals, Cambridge, Ontario, fetal bovine serum (FBS) from HyClone, Logan, UT, western blot cell lysis buffer from Cell Signaling Technology, Beverly, MA.

II. BUFFERS

PUCS solution :	0.01 M EDTA, 0.14M NaCl, 4.2 mM $NaHCO_3$, 6 mM KCl.
Phosphate buffered saline (PBS):	0.14 M NaCl, 3.6 mM KCl, 2 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 0.9 mM $CaCl_2$-H_2O, 0.5 mM $MgCl_2$-6 H_2O in H_20, pH 7.2, filtered.
Tris buffered saline (TBS):	0.02 mM Tris base, 0.14 M NaCl, 3.8 mL 1 N HCl in 1 L of H_2O.
Red blood cell lysis buffer:	0.15 M NH_4Cl and 17 mM Tris in water, pH 7.2.

5X first strand buffer:	375 mM KCl, 250 mM Tris-HCl and 15 mM $MgCl_2$.
PCR buffer:	200 mM Tris-HCl (pH 8.4) and 500 mM KCl.
Western blot cell lysis buffer:	20 mM Tris-HCl (pH7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na_3VO_4, 1 μg/mL leupeptin.
SDS loading buffer:	187.5 mM Tris-HCl (pH6.8), 6% SDS, 30% glycerol, 0.03% phenol red, 10% DTT (1.25 M).
10% acrylamide gel:	5 mL 1.5 M Tris-Cl pH 8.8, 10.3 mL dd H_2O, 5 mL 40% acrylamide (29:1), 200 μL 10% SDS, 18 μL tetramethylethylenediamine (TEMED).
Transfer buffer:	50 mM Tris, 40 mM glycine, 1.3 mM SDS, 20% methanol.

III. ANIMALS

Adult male Balb/cCr mice were obtained from HSLAS (Health Sciences Laboratory Animal Services), University of Alberta. Adult male C57BL/6J mice and Sprague Dawley rats were purchased from Charles River (St -Constant, Quebec). Animals were fed and watered *ad libitum*, and maintained on a 12 h light/dark cycle

in a virus antibody-free facility. Animals were allowed to acclimatize for 1 wk after shipping to reduce stress levels. All experimental procedures were approved by the University of Alberta Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

IV. ISOLATION OF CELLS FROM THE LUNGS AND FROM SPLEEN

Animals were anaesthetized by intraperitoneal injection of 1:1 mixture of Rompun (20 mg/mL) and Ketalean (100 mg/mL). The volume injected was of 200 μL for mice and 1 mL for rats. The trachea was exposed by blunt dissection and a polyethylene catheter attached to a 21 G ½ (mouse) or 18 G (rat) needle was introduced. Because the presence or absence of Ca^{2+} and Mg^{2+} ions influences adherence of cells, EDTA, a chelating agent, was added to the lavage fluid. The lungs were washed with cold sterile PBS (pH 7.2) containing 0.2 mM EDTA; a total of 8 mL in eight 1 mL aliquots (mouse) and a total of 50 mL in five 10 mL aliquots (rat) of this solution was gently massaged into the lungs and over 90% of the fluid was retrieved. Cells were kept on ice, centrifuged at 200 x g for 10 min at 4°C and resuspended in PBS. On average, 3.4×10^5 cells were obtained from each mouse (Table 5 in Result section). Differential counts were made from cytospins stained with Diff quick. The BAL cells contained morphologically identifiable large (> 94%) and smaller cells. Macrophages have often cytoplasmic vacuoles and the cell surface is ruffled and not as refractile as that of lymphocytes. An average of 250 ± 135 cells

were counted in 20 different samples and Table 6 in the Result section summarizes differential cell counts of BAL fluid recovered from Balb/c and C57Bl/6 mice.

The spleen was also removed and kept on ice. Splenic tissue was disrupted using a Dounce homogenizer and cells were washed in PBS, spun at 200 x g for 5 min at 4°C. Cells were resuspended in 5 mL of cold red blood cell lysis buffer and kept on ice for 1 min. Red cell lysis was stopped by adding 40 mL of cold PBS. Cells were washed at 200 x g for 5 min at 4°C and resuspended in PBS.

V. CELL LINES

The adherent mouse peritoneal macrophage (PM) cell line RAW 264.7 and an adherent mouse AM cell line, MH-S (18-19), were a generous gift from C. Mowat and Dr. B. Winston (University of Calgary). BW5147 cells transfected with mouse CD8α chain were provided by Dr. John Elliott (University of Alberta). P388D1 and J774A.1 cell lines are adherent mouse macrophage lines provided by Dr. Hanne Ostergaard (University of Alberta).

MH-S cells were grown in complete RPMI 1640 containing 2 mM L-glutamine, 1% penicillin/streptomycin and 10% heat-inactivated (at 56°C for 30 min) FBS. RAW 264.7 cells were grown in high glucose (4.5 g/L) DMEM supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin, 1 mM sodium pyruvate, 1.5 g/L $NaHCO_3$, 10% FBS and 50 mM βME. BW5147 cells were grown in DMEM with 4 mM L-glutamine adjusted to contain 1.5 g/L $NaHCO_3$, 4.5 g/L glucose, and 10% FBS. P388D1 were grown in RPMI 1640 medium with 2 mM L-glutamine, 1.5 g/L $NaHCO_3$, 4.5 g/L glucose, 10 mM HEPES, 1%

penicillin/streptomycin, 1.0 mM sodium pyruvate, and 10% FBS. J774A.1 cells were grown in DMEM with 4 mM L-glutamine, 1.5 g/L $NaHCO_3$, 1% penicillin/streptomycin, 4.5 g/L glucose, and 10% FBS. All media were adjusted to a pH 7.4. Cells were grown to confluence at 37°C in an incubator with 5% CO_2 for MH-S, P388D1 and J774A.1 cells and 7.5% CO_2 for RAW 264.7 cells. The cells were removed from the flask with PUCS solution using a cell scraper. Cells were washed in cell culture medium, centrifuged at 100 x g for 5 min at room temperature and resuspended in their respective growth medium or PBS.

VI. PRIMER DESIGN

Primer sets for b-actin, CD3 epsilon chain and CD8α and β chains were designed and compared to known gene sequences using GENBANK of the National Center of Biotechnology Information (NCBI) and were used in reverse transcriptase (RT)- polymerase chain reaction (PCR) experiments. The mapping of the regions of CD8α and CD8β chains amplified by each pair of primers is illustrated in Figure 2. CD8 primers sequences, optimal annealing temperatures and expected product sizes obtained with these primers are listed in Table 3.

For the α chain, the first primer pair was designated 1α and includes the cytoplasmic tail domain of CD8α chain mRNA. It gives a product of 727 base pairs (bp), out of a total length of 744 bp for the entire α chain mRNA (23). The second primer pair called 3α amplifies the cDNA from the leader domain to the end of the immunoglobulin (Ig)-like domain and gives a product of 432 bp. The third pair of

primers, 4α, spans the hinge region to the cytoplasmic domain and gives a product of 316 bp. Finally, the fourth primer pair, 5α, includes the leader domain, the Ig-like domain and the hinge region of CD8 mRNA and results in a 598 bp product.

The total length for the entire β chain mRNA is 1111 bp (120). For the β chain, the first pair of primers, 1β, amplifies the coding sequence of CD8 β from the Ig-like domain to the transmembrane domain. The second primer pair, 2β, includes part of the Ig-like domain, the hinge and transmembrane regions. These primer pairs result in products of 605 bp and 344 bp respectively.

VII. RNA EXTRACTION

Total RNA from spleen, BAL and cell lines was isolated using TRIzol reagent according to the manufacturer's instructions. Between 1 and 3 million cells were resuspended in 1 mL TRIzol reagent. After 5 min at room temperature, 0.2 mL of chloroform was added and tubes were gently inverted 5 times. The mixture was then incubated at room temperature for 3 min and centrifuged at 12,000 x g for 15 min at 4°C. The aqueous upper phase containing RNA was transferred to a clean tube. Isopropanol (0.5 mL) was added to precipitate the RNA. After an incubation of 10 min at room temperature, the mixture was centrifuged at 12,000 x g for 10 min at 4°C. The resulting RNA pellet was washed in 1 mL of 70% ethanol, air-dried, and dissolved in RNAse treated water. It was then incubated for 10 min at 55°C to increase solubility. Absorbance values were measured in a spectrophotometer at 260 nm to determine the final RNA concentration.

VIII. REVERSE-TRANSCRIPTION AND POLYMERASE CHAIN REACTION

RNA (1 μg) and 1 μl of oligo(dT) (0.5 μg/μL) were mixed with RNAse free water resulting in a total reaction volume of 11 μL. The mixture was heated to 70°C for 10 min on a thermal cycler (PTC-100 Programmable Thermal Controller, MJ Research, Reno, NV). The samples were reverse-transcribed by using M-MLV reverse transcriptase enzyme according to the manufacturer's instructions. The RT master mix containing 4 μL of 5X first strand buffer, 2 μL of 0.1 M DTT, 1 μL of mixed dNTP's (10 mM), 1 μL of RNAse free water and 1 μL of M-MLV enzyme, was added to the RNA mixture. The sample was incubated at 37°C for 1 h and 95°C for 5 min to inactivate the enzyme.

The PCR amplification was performed as follows. A total of 2 μL of cDNA product was added to a tube containing PCR buffer, 1.5 mM $MgCl_2$, mixed dNTP's (1.25 mM each), 0.5 μL Taq DNA polymerase, primer pairs (20 μM) and RNAse free water, resulting in a total reaction volume of 20 μL. The primers used for cDNA amplification are listed in Table 3. Optimization of annealing temperatures and cycle numbers was performed using splenic cDNA. Samples were heated to 94°C for 3 min and amplified for 40 cycles (94°C for 30 s, 46°C for 1 min, 72°C for 30 s) for CD8 α chain and β chain and 40 cycles (94°C for 30 s, 50°C for 1 min, 72°C for 30 s) for CD3 with a common final step at 72°C for 7 min. β-actin was used as a housekeeping gene. Products were electrophoretically separated on a 2% agarose gel,

stained with ethidium bromide (EtBr) and visualized by ultraviolet light. The product size was determined using a 2 Kb bp ladder.

Table 3: PCR Primers Sequence And Annealing Temperatures

A)

Primer pairs	Upstream primer (5' to 3')	Downstream primer (5' to 3')
B-actin	CCA TGT ACG TAG CCA TCC A	GAT AGA GCC ACC AAT CCA C
CD8 1 α (7-734 bp)	TCA CCG TTG ACC CGC TTT CT	TTC TCT GAA GGT CTG GGC TT
CD8 3 α (7-439 bp)	TCA CCG TTG ACC CGC TTT CT	AAG GAC TGG CAC GAC AGA AC
CD8 4 α (418-734 bp)	GTT CTG TCG TGC CAG TCC TT	TTC TCT GAA GGT CTG GGC TT
CD8 5 α (7-605 bp)	TCA CCG TTG ACC CGC TTT CT	CCA AGG GTG CCC AGA TGT AA
CD8 1 β (70-675 bp)	GGT CTT CAG TAT GAA GCT GG	TTC ATG AAG TGA ATT CGG GC
CD8 2 β (331-675 bp)	TTC AGA CTC AAG ACG GCC CT	TTC ATG AAG TGA ATT CGG GC
CD3 epsilon	AAT GGC CAA GAG CTG CCT CAG AAG	GAC TGC TCT CTG ATT CAG GCC AGA

B)

Primer pairs	Product size	Annealing T°
B-actin	650 bp	46°C
CD8 1 α (7-734 bp)	727 bp	46°C
CD8 3 α (7-439 bp)	432 bp	46°C
CD8 4 α (418-734 bp)	316 bp	46°C
CD8 5 α (7-605 bp)	598 bp	46°C
CD8 1 β (70-675 bp)	605 bp	46°C
CD8 2 β (331-675 bp)	344 bp	46°C
CD3 epsilon	404 bp	50°C

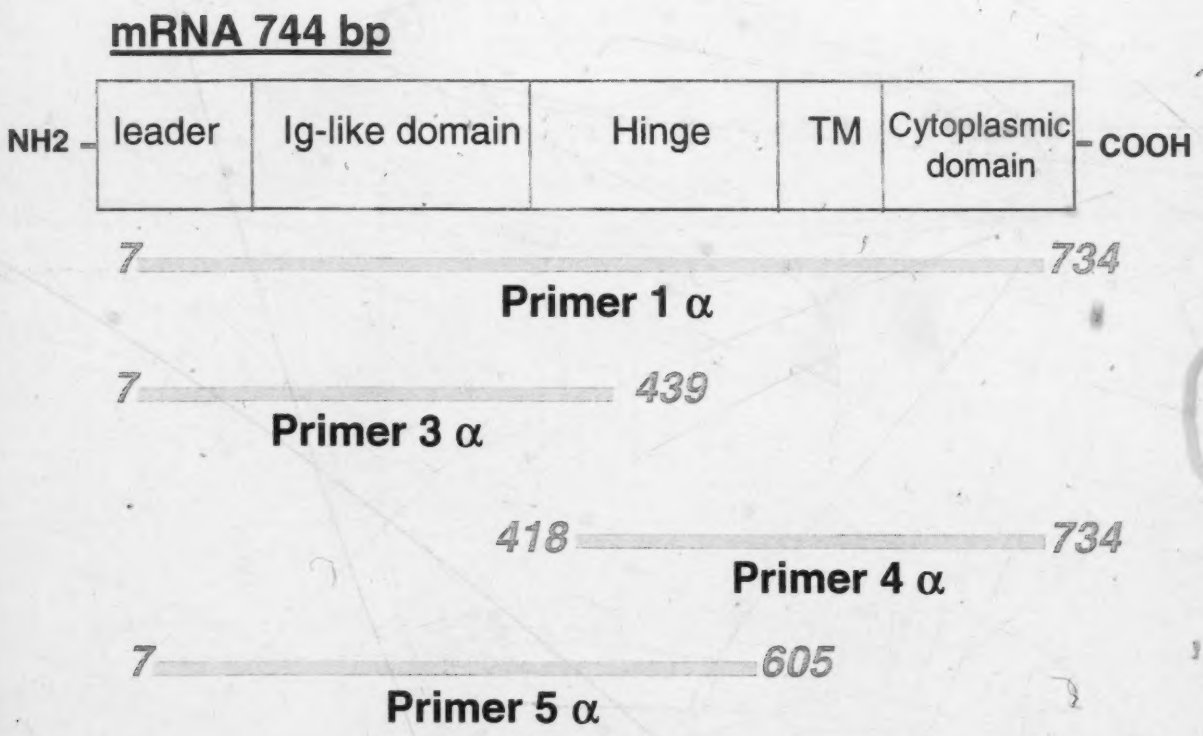


Figure 2 a) Mouse CD8 Alpha Chain Primer Pairs
Primer pair 1α : 7-734 bp
Primer pair 3α : 7-438 bp
Primer pair 4α : 418-734 bp
Primer pair 5α : 7-605 bp

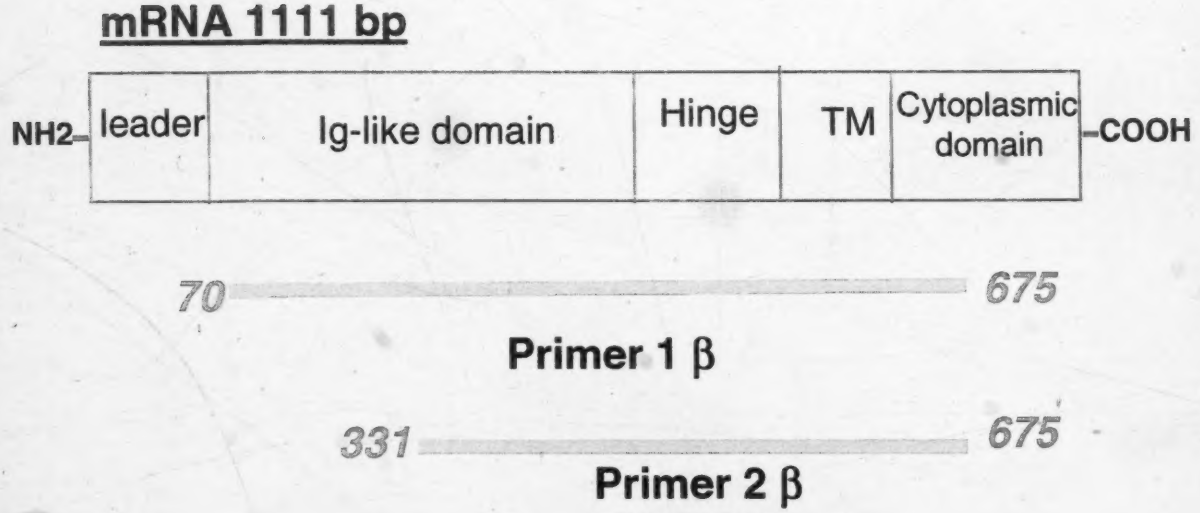


Figure 2 b) Mouse CD8 Beta Chain Primer Pairs
Primer pair 1β: 70-675 bp
Primer pair 2β: 331-675 bp

IX. CLONING OF PCR PRODUCTS

Cloning was performed using a modified Invitrogen's T/A Cloning® protocol with the pCR®2.1 vector. All solutions were provided by the manufacturer. The PCR product/plasmid ligation reaction was carried out in a tube containing 2 μL of PCR product, 1 μL 10X ligation buffer, 2 μL plasmid pCR®2.1, 1 μL T4 DNA ligase, 4 μL of RNAse free water and was incubated at 14°C overnight in a thermal cycler. Two μL of 0.5 M βME and 2 μL of ligation solution containing the ligated PCR product were added to INVαF' (*Escherichia coli*) cells. The vials were incubated on ice for 30 min. Samples were heated to 42°C in a water bath for 30 s and placed on ice for an additional 2 min. SOC medium (250 μL) was then added to each tube and the transformation vials were shaken horizontally at 37°C for 1 h at 225 rpm. The samples were spread on agar plates supplemented with X-Gal (2 mg), IPTG (4 mM) and ampicillin (50 μg/mL), and were incubated at 37°C overnight.

Four different white colonies, which represent cells that have incorporated the plasmid with the DNA insert, were picked and inoculated onto a second plate to verify the phenotype of these colonies. White isolated colonies were grown in 5 mL of LB broth containing ampicillin (50 μg/mL) and incubated at 37°C for 2 d. Plasmid DNA was isolated using the Genelute™ Plasmid Miniprep kit system according to the manufacturer's instructions. Cells were centrifuged at 500 x g for 10 min and resuspended in 200 μL resuspension solution. Lysis buffer (200 μL) was added and mixed by inversion of the tube. Lysis was stopped by adding 350 μL of

neutralizing solution after the mixture turned clear or after a maximum of 5 min. Cell debris was pelleted by centrifugation at 12,000 x g for 10 min.

The lysate was transferred to a column and centrifuged at 12,000 x g for 30 s. The column was washed with 750 μL of wash solution by centrifugation at 12,000 x g for 30 s and dried by an additional centrifugation step at 12,000 x g for 1 min. The column was transferred to a clean tube and RNAse free water was added to the column to elute the DNA. After centrifugation at 12,000 x g for 1 min, the eluate containing the DNA was transferred to a clean tube and quantified by measuring absorbance at 260 nm and 280 nm in a spectrophotometer. Product size was determined after electrophoresis on a 2% agarose gel.

The cDNAs were sequenced with M13 forward and reverse primers using the dye terminator cycle sequencing kit (Perkin Elmer, Foster City, Ca) in the DNA core lab of the University of Alberta. The nucleotides sequences were analyzed by Basic Local Alignment Search Tool (BLAST) to determine the degree of homology with the published sequence found in the NCBI nucleotide databases.

X. SINGLE-CELL RT-PCR PROCEDURE

Balb/c mouse lungs were lavaged as described above (n=3). The BAL cells as well as the cell lines were resuspended in PBS at a concentration of $1x10^6$ cells/mL. A standard patch clamp set up was used to collect individual cells. A borosilicate-patch pipette was filled with 5 μL of RNAse free water and the cell of interest was aspirated resulting in cell lysis. The cells were chosen on the basis of their macrophage-like morphology, characteristic under a light microscope. The

biggest cells with abundant cytoplasm showing complexity and presence of vacuoles were aspirated. The pipette content was transferred into a PCR tube containing 5 µL of RNAse free water, 0.5 µL of 0.1 M DTT, 0.5 µL RNAse inhibitor (40 U/µL) and 1 µL of oligo(dT) (500 mg/mL). The mixture was heated to 70°C for 10 min before adding 2 µL of PCR buffer, 2 µL of $MgCl_2$ (25 mM), 1 µL mixed dNTPs (10 mM), 0.5 µL of RNAse inhibitor (40 U/µL) and 1 µL of Superscript II (200 U/µL). The sample was heated to 37°C for 1 h and to 95°C for 5 min to complete the transcription step. Each sample was tested by PCR for the expression of CD8 and CD3 using 6 µL of cDNA, and β-actin using 4 µL of cDNA. All products were electrophoresed on a 2% agarose gel, stained with EtBr, and product sizes were determined using ultraviolet light.

XI. FLOW CYTOMETRY

Expression of CD8 on the cell surface was studied on mouse BAL and spleen cells, rat spleen cells, and the cell lines Raw 264.7 and MH-S. All antibodies (Ab) were used at a concentration of 5 µg/ml. Ab used are listed in Table 4.

Normal mouse serum (10 %) was added to 5 X 10^5 cells and incubated for 30 min at 4°C in a 96-well U-bottom plate. Thereafter, 100 µL of Ab obtained from hybridoma supernatants or purified Ab were added to the wells and incubated for 1 h at 4°C. Cells were then washed twice with 200 µL PBS before adding the secondary Ab (dilution 1:50). Corresponding isotype control Ab were used to calibrate the flow cytometer. Cells were incubated for 1 h at 4°C, then washed 3 X with PBS and

finally resuspended in 300 μL of 3% formalin. The samples were protected from light until measured. Unlabelled cells were used for size calibration.

XII. WESTERN BLOT

Protein isolation

Cells were resuspended in cell lysis buffer and kept on ice for 20 min. The samples were centrifuged at 8,000 x g for 10 min and the supernatant was stored at -80°C. Protein content from rat and mouse spleen cells and AM, NR8383, Raw 264.7 and MH-S cell line samples, was assessed using the DC protein assay kit by measuring the absorbance of the sample at 690 nm and comparing it to a standard curve. Before loading the samples, the supernatant was diluted 1:3 with SDS loading buffer and boiled for 3 min.

Electrophoresis/Transfer/Detection

Samples and molecular weight protein markers were loaded on a 10% SDS polyacrylamide gel. The proteins were then transferred onto a PVDF membrane for 1 h at room temperature using a semi-dry transfer cell (BioRad) with transfer buffer. The membranes were blocked in 5% milk/TBS-Tween (0.2%) overnight on a rocker at 4°C. Ab used by Western blot are listed in Table 4. Unconjugated primary Ab diluted 1:1000 in blocking solution were then incubated with the membranes for 1 h at room temperature followed by two washes of 10 min and two washes of 5 min with TBS-Tween (0.2%). To reduce low affinity protein binding, membranes stained with OX-8, sc-7970 and sc-7188 Ab were washed an additional time with a 3 M urea

solution for 5 min before starting the wash protocol described above (121,122). A horseradish peroxidase-conjugated secondary Ab also diluted (1:10,000) in blocking solution was incubated with the membranes for 45 min at room temperature. The membranes were washed as described above and protein bands were detected using enhanced chemiluminescence. Band sizes were estimated by comparison with the molecular weight standards using the SigmaGel program (Jandel Scientific, San Raphael, CA).

Table 4: Primary, Secondary And Isotype Control Ab

A) Anti- CD8 alpha chain

Species	Name	Isotype	Form	Company	Catalogue #	Flow cytometry	Western blot
Mouse	2.43	rat IgG2	Sup	gift *	/	✓	
	2.43	rat IgG2	Pur	gift *	/	✓	✓
	53-6.7.2	rat IgG2b κ	Sup	gift *	/	✓	
	53-6.7.2	rat IgG2b κ	Pur	Pharmingen	01041A	✓	✓
	KT 15	rat IgG2a	FITC	Harlan Sera-lab	FMAS 747p	✓	
	YTS-105.18	rat IgG2a	Pur	Harlan Sera-lab	MAS 748p	✓	✓
	YTS-169.4	rat IgG2b	FITC	Cedarlane	CL169F	✓	
	116-13.1	mouse	Sup	gift [1]	/	✓	
	D-9	mouse IgG2a	Pur	Santa Cruz	sc-7970		✓
	H-160	rabbit IgG	Pur	Santa Cruz	sc-7188		✓
Rat	G28	mouse IgG2a	Pur	Pharmingen	559977	✓	✓
	OX8	mouse IgG1	Pur	Pharmingen	22071D	✓	✓
	R1-1085	mouse IgG2a	Pur	Seikagaku	270456	✓	✓
Human	LT8	mouse IgG1	Pur	Serotec	MCA1226	✓	

* From Dr. Kevin Kane, Department of Microbiology and Immunology, University of Alberta

[1] From Dr. Hanne Ostergaard, Department of Microbiology and Immunology, University of Alberta

Sup: Hybridomas supernatants;Pur: purified antibody; FITC: Fluorescein isothiocyanate

Table 4: Primary, Secondary And Isotype Control Ab

B) Anti- CD8 beta chain

Species	Name	Isotype	Form	Company	Catalogue #	Flow cytometry	Western blot
Mouse	YTS-156-7	rat IgG2b	Sup	gift *	/	✓	
	53-5.8.3	rat IgG1 κ	Pur	Pharmingen	01051D	✓	✓
	CT-CD8b	rat IgG2a	FITC	Serotec	MCA1362F	✓	
Rat	341	mouse IgG1	Pur	Pharmingen	554971	✓	✓

* From Dr. Kevin Kane, Department of Microbiology and Immunology, University of Alberta

C) Isotype control Ab

Isotype	Form	Company	Catalogue #
mouse IgG1	Pur	Pharmingen	557273
normal rat IgG	Pur	Santa Cruz	sc-2026
normal goat IgG	Pur	Santa Cruz	sc-2028
normal rabbit IgG	Pur	Santa Cruz	sc-2027
mouse IgG2a	Pur	Pharmingen	71151A

D) Secondary Ab

Host	Form	Company	Catalogue #	Recognition
goat	HRP	Santa Cruz	sc-2054	rabbit IgG
goat	HRP	Cedarlane	CLCC40007	rat IgG (H+L)
donkey	HRP	Santa Cruz	sc-2056	goat IgG
goat	HRP	Cedarlane	CLCC30207	mouse IgG (H+L)
donkey	FITC	Serotec	Star88F	sheep/goat IgG
goat	FITC	Serotec	Star69	rat IgG
goat	FITC	Medicorp	AM14708	mouse IgG+IgM

HRP: Horseradish peroxidase

XIII. MONOCLONAL Ab PRODUCTION

In the absence of an Ab against the mouse CD8α hinge region, which is the epitope region detected by OX-8 monoclonal Ab (mAb), mAb were raised against mouse CD8α hinge region by Dr. Terry Pearson, University of Victoria.

Peptide design

The mouse CD8 hinge region sequence was analyzed by computer to determine the predicted region(s) most likely to be recognized by B cells. From a Jameson-Wolf plot for antigenic index (123), one region for mouse CD8 was chosen and the sequence of this region was used to design a peptide. The mouse peptide (15-mer) was KVNSTTTKPVLRTP. A cysteine residue was added to the peptide to the C-terminal to allow coupling of the peptide to keyhole limpet hemacyanin (KLH) and to bovine serum albumin (BSA). The KLH conjugates were used in the immunization step and the BSA conjugates were necessary for the screening of the hybridomas supernatants by ELISA.

Immunization

A rapid immunization method was used for hybridoma production. For each antigen, at intervals of three days, two female BALB/c mice were given a total of five subcutaneous bilateral injections of 50 μg of KLH-peptide conjugate in a total volume of 50 μl of physiological saline per leg in the diffuse adipose tissue between the calcaneous tendon and the posterior aspect of the tibia. Three days after the last injection, the draining popliteal, inguinal and femoral lymph node cells were

collected, teased into a single cell suspension and fused with X63-Ag8.6.5.3 parental myeloma cells. The immunization procedure and cell fusion was performed as described previously (124).

Selection and cloning of hybridomas

Single step selection and cloning of hybridomas was then performed using the ClonaCell-HY™ system (StemCell Technologies Inc., Vancouver, B.C.) following the instructions of the manufacturer. Essentially, the cell fusion mixture was diluted and plated in a semi-solid methylcellulose containing HAT selective medium and B-cell growth factors, allowing a single-step selection and cloning of the hybridomas. After 10-14 days of growth, 572 anti-mouse peptide and 420 anti-human peptide hybridomas were selected and grown in individual wells of 96 well tissue culture plates.

Enzyme-linked immunosorbent assays

After 3-4 days, the hybridoma tissue culture supernatants were screened by indirect enzyme-linked immunosorbent assay (ELISA) on solid-phase adsorbed antigen (1 μg/well of BSA-peptide). Monoclonal antibodies in hybridoma supernatants were screened for binding to BSA-peptide conjugates by indirect ELISA (125). Purified peptide conjugates were dried at 1 ug per well onto flat-bottom ELISA plates (Falcon 3915 PRO-BIND™ Assay Plates, Becton-Dickinson, Oxnard, CA). After screening by ELISA, stable hybridoma clones secreting peptide-specific mAb (135 anti-human peptide and 136 anti-mouse peptide) were chosen for further

testing. Supernatnants from these were screened on human transferrin in order to eliminate "sticky" antibodies. The remaining hybridoma clones were grown up in 50 ml tissue culture flasks, the supernatants were rescreened 10 weeks later in ELISA and those remaining positive were selected for cryopreservation and further testing.

XIV. STATISTICS

All values are given as mean ± standard deviation (SD) for the number of cells obtained.

CHAPTER 3

RESULTS

I. Bronchoalveolar Lavage

BAL samples were obtained from Balb/c and C57Bl/6 mice. An average of 3.4 x 10^5 cells were harvested per mouse (Table 5). Differential cell counts for 20 different Balb/c and 3 different C57Bl/6 BAL samples stained with Diff quick are listed in Table 6. Over 94% of the cells recovered from BAL fluid had morphological characteristics typical of macrophages under a light microscope.

Table 5: Number Of Cells From BAL

Samples	# of mice	# of cells (M)	cells/mouse x 10^5
1	10	3.6	3.6
2	10	3.0	3
3	10	2.2	2.2
4	12	3.6	3
5	16	7.2	4.5
6	16	5.9	3.7
7	13	2.5	1.9
8	10	5.3	5.3
9	9	3.3	3.6
10	19	10.5	5.5
11	10	5.0	5
12	6	3.2	5.3
13	8	3.3	4.1
14	8	2.1	2.6
15	8	1.3	1.6
16	10	2.8	2.8
17	11	2.4	2.2
18	10	2.1	2.1
19	3	1.1	3.5
20	10	3.3	3.3
Average			**3.4 ± 1.2**

Table 6: Differential Cell Counts Of BAL Fluid (n=20)

Strain	Cell types	Mean %	SD
Balb/c	Macrophage	97.7	1.5
	Lymphocytes	2.0	2.0
	Neutrophils	0.3	0.4
C57Bl/6	Macrophage	94.0	1.4
	Lymphocytes	4.0	0.9
	Neutrophils	3.6	1.8

SD: standard deviation ± values

II. Analysis of CD8 expression in Balb/c mice BAL cells

A. PCR analysis of CD8α and CD8β in splenic and BAL cells

PCR analysis was conducted on cells from BAL samples for the expression of CD8 mRNA and mouse splenic cells were used as a positive control. The results indicate that mouse spleen tissue expressed CD8α and CD8β mRNA (Figure 3). The products obtained with the primer pairs 1α, 3α, 4α, 5α, 1β and 2β were of the predicted sizes and sequence analysis confirmed their identity. The comparison between the PCR product obtained with primer pair 3α and the published sequence for mouse CD8α showed that the product shared 99% identity with mouse CD8 (Figure 4). BAL cells expressed mRNA for CD8α and CD8β as shown in Figure 5. Using the same concentration of mRNA, the bands intensities for mouse spleen and BAL cells were comparable except for primer pair 1α and primer pair 5α. No PCR products were detected when the RT step was omitted suggesting the absence of genomic DNA in our sample.

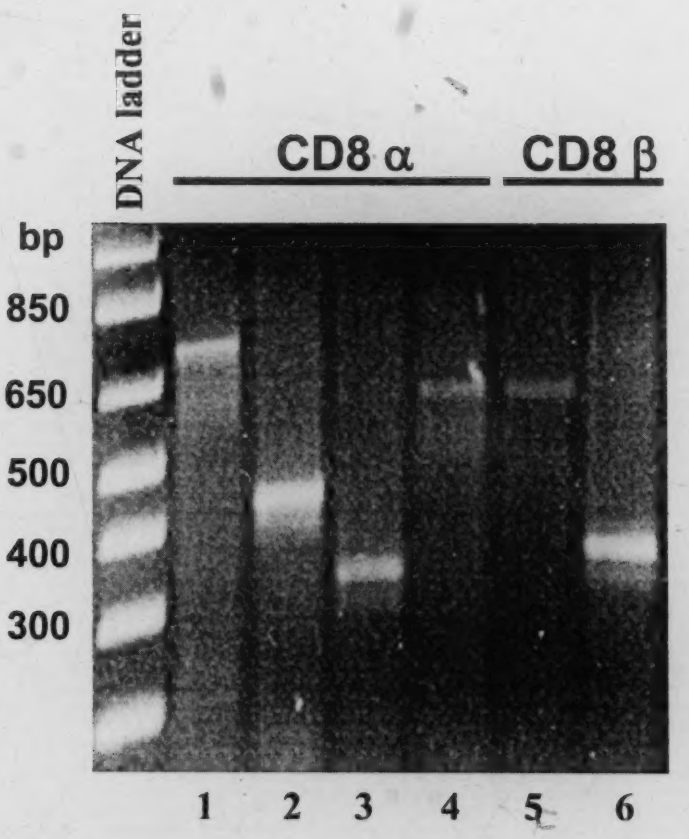


Figure 3: CD8α and CD8β mRNA expression in splenic cells using different primer pairs. Lane 1: primer pair 1α; lane 2: primer pair 3α; lane 3: primer pair 4α; lane 4: primer pair 5α; lane 5: primer pair 1β; lane 6: primer pair 2β.

```
S 3α(1–3):       aaggactggcacgacagaactgaagtacatcaccgagttgctgatgactgagcagaaa
mCD8:      564 aaggactggcacgacagaactgaagtacatcaccgagttgctgatgactgagcagaaa 507

S 3α(1):         tagtagccttcgttttccttgctgaacttgttcagggtgagaacgtacttacttattcgtgtcc
S 3α(2–3):       tagtagccttcgttttccttgctgaacttgttcagggtgagaacgtactta ttattcgtgtcc
mCD8:      506 tagtagccttcgttttccttgctgaacttgttcagggtgagaacgtactta ttattcgtgtcc 444

S 3α(1–3):       ctcatggcagaaaacagtttcgacgaattcagcttctcgtcccacgttatcttgttgtggga
mCD8:      443 ctcatggcagaaaacagtttcgacgaattcagcttctcgtcccacgttatcttgttgtggga 381

S 3α(1):         tgaagccatatagacaacgaaggtgggctgggggagt t t ggagctggagttctggaa
S 3α(2):         tgaagccatatagacaacgaaggtgggctgggggagtnntggagctggagttctggaa
S 3α(3):         tgaagccatatagacaacgaaggtgggctgggggagt t tggagctggagttctggaa
mCD8:      380 tgaagccatatagacaacgaaggtgggctgggggagt t tggagctggagttctggaa 324

S 3α(1):         gagcc aagagcatccttgcgaaacggacccccaacacttcacataccaggtccaccttc
S 3α(2):         gagcct aagagcatccttgcgaaacggacccccaacacttcacataccaggtccaccttc
S 3α(3):         gagcc aagagcatccttgcgaaacggaccnnaacacttcacataccaggtccaccttc
mCD8:      323 gagcc aagagcatccttgcgaaacggacccccaacacttcacataccaggtccaccttc 266

S 3α(1):         ctgaccaagttcggcgtccattttctttggaaagattnggagttcgggtgcctgtggcttag
S 3α(2):         ctgaccaagttcggcgtccattttctttggaaagattcggagttcgggtgcctgtggcttag
S 3α(3):         ctgaccaagttcggcgtccattttctttggaaagattcggagttcgggtgcctgtggcttag
mCD8:      265 ctgaccaagttcggcgtccattttctttggaaagattcggagttcgggtgcctgtggcttag 204

S 3α(1):         ctt ctccactccccaggataatcgactcannccagcagcagcaggttcagcgacagaa
S 3α(2):         ctt ctccactccccaggataatcgactcac  ccagcagcagcaggttcagcgacagaa
S 3α(3):         ctnctccactccccaggataatcgactcac  ccancagcagcaggttcagcgacagan
mCD8:      203 ctt ctccactccccaggataatcgactcac  ccagcagcagcaggttcagcgacagaa 147


S 3α(1–3):       agcgggtcaacggtga
mCD8:      146 agcgggtcaacggtga 131
```

Figure 4: Comparison of the products obtained with primer pair 3α on spleen (S 3α 1, 2, 3; if only 2 sequences are compared, sequences from S1, S2 and S3 were identical) with the published sequence for the mouse CD8α (mCD8; 131-564 bp) gene sequence (23). Results were obtained with products from three separate RNA preparations.

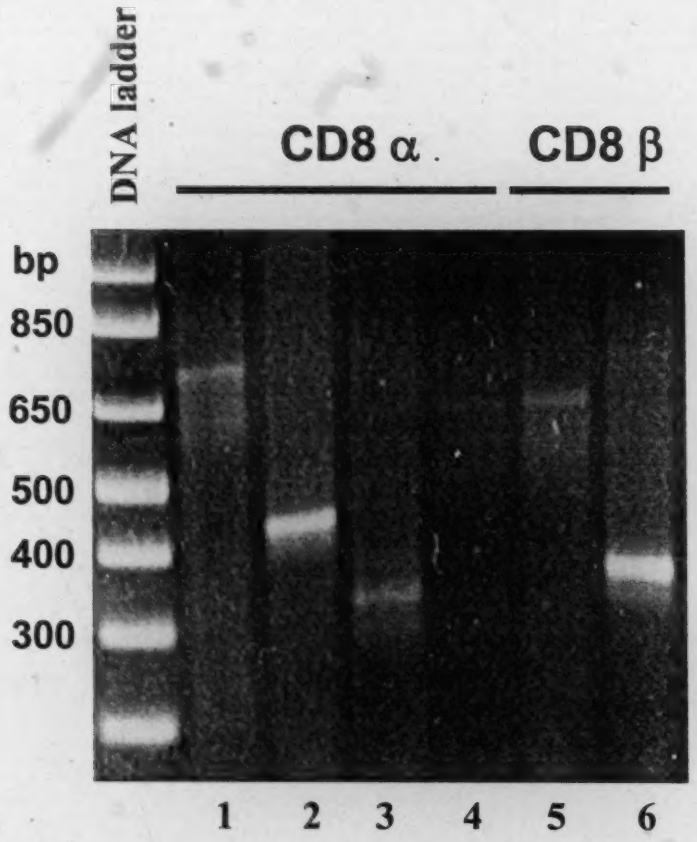


Figure 5: CD8α and CD8β mRNA expression in Balb/c BAL cells using different primer pairs. Lane 1: primer pair 1α; lane 2: primer pair 3α; lane 3: primer pair 4α; lane 4: primer pair 5α; lane 5: primer pair 1β; lane 6: primer pair 2β.

```
M 5α(1–2) :        ttgacccgctttctgtcgctgaacctgctgctgctgggtgagtcgattatcctggggagt
mCD8:       139 ttgacccgctttctgtcgctgaacctgctgctgctgggtgagtcgattatcctggggagt 198

M 5α(1–2) :        ggagaagctaagccactggcacccgaactccgaatctttccaaagaaaatggacgcc
mCD8:       199 ggagaagctaagccacaggcacccgaactccgaatctttccaaagaaaatggacgcc 255


M 5α(1) :          gaacttggtcagaaggtggacctggtatgtgancgtgttggggtccgtttcgcaaggat
M 5α(2) :          gaacttggtcagnaggtggacctggtatgtgaa gtgttggggtccgtttcgcaaggat
mCD8:       256 gaacttggtcagaaggtggacctggtatgtgaa gtgttggggtccgtttcgcaaggat 314


M 5α(1) :          gctcttggctcttccagaactccagctccaaactcccccagcccaccttccgttgtctata
M 5α(2) :          gctcttggctcttccagaactccagctccaaactcccccagcccaccttcgttgtctata
mCD8:       315 gctcttggctcttccagaactccagctccaaactcccccagcccaccttcgttgtctata 376


M 5α(1) :          tggcttcatcccacaaccaagataacgtgggacgagaagctgaatccgtcgaaactgt
M 5α(2) :          tggcttcatcccacaa caagataacgtgggacgagaagctgaattcgtcgaaactnt
mCD8:       377 tggcttcatcccacaa caagataacgtgggacgagaagctgaattcgtcgaaactgt 433


M 5α(1) :          tttctgcccatgagggacacgaataataagtacgttctcaccctgaacaagttcagcaag
M 5α(2) :          tttctg ccatgatggacacgaataataagtacgttctcaccctgaaccagttcagcaag
mCD8:       434 tttctg ccatgagggacacgaataataagtacgttctcaccctgaacaagttcagcaag 492


M 5α(1) :          gaaaa cgannggctactatnttctgctcagtnatc
M 5α(2) :          nnnaacga ag nctactat ttcngctcagtcatc
mCD8:       493 gaaaacga aggctactat ttctgctcagt catc 526
```

Figure 6: Comparison of the product obtained with primer pair 5α for BAL cells (M 5α 1and 2; if only 2 sequences are compared, M1 and M2 were identical) with the published sequence for the mouse CD8α (mCD8; 139-526 bp) gene sequence (23). Results were obtained with products from two different RNA preparations.

Three PCR products obtained with primer pairs 3α, 5α and 1β from independent preparations of RNA were cloned and sequenced. The sequences were compared to the published gene sequence for mouse CD8. Macrophage CD8, as amplified by the 5α primer pair, shared 97% identity with mouse T cell CD8 (Figure 6). Some of the PCR products obtained with primer pairs 3α and 5α (Figure 4 and 6) showed additional nucleotides compared to the CD8 published sequence. This could be attributed to a technical error but whether the additional nucleotides were present in both strands could not be verified. It would have been valuable to sequence more PCR products to determine reproducibility and to evaluate whether the addition of nucleotides led to a frame shift, thus possibly altering protein translation.

B. Comparison of CD3 and CD8 expression in BAL cells

To determine whether the positive PCR results for CD8 expression in BAL cells could be attributed to contaminating cells present in BAL fluid, particularly in T lymphocytes, different RNA preparations were tested for CD3 epsilon subunit and CD8 expression. Figure 7 shows 12 different RNA samples (lane 1-12). Samples 1, 3, 4, and 6 were positive for CD3 and CD8. Samples 2 and 7 were positive for CD8 only. The reminder of the samples were either positive for CD3 only (5, 9, 10 and 12) or negative for both markers (8 and 11). Table 7 summarizes the results for all samples tested. Thirty BAL samples (n=2) were studied for the expression of CD8 and CD3. Sixteen samples expressed CD3 and CD8, five were $CD3^+$ $CD8^-$, 5 were positive for CD8 but not CD3 and 4 were negative for both markers.

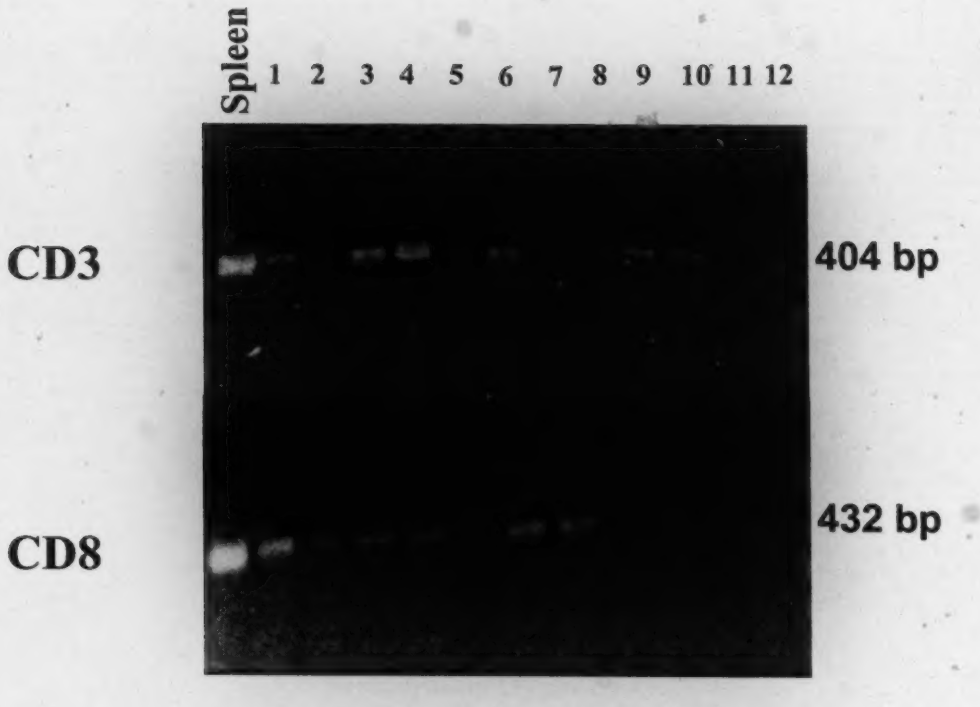


Figure 7: mRNA expression of CD3 (top panel) and CD8 (bottom panel) in different BAL samples (lanes 1-12). Splenic cells were used as a positive control.

Table 7: CD3 And CD8 Expression In BAL Samples

Total number of BAL samples: 30

Markers	CD8+	CD8-
CD3+	16	5
CD3-	5	4

C. Single-cell RT-PCR analysis

To confirm that the PCR results using BAL cells reflected CD8 expression by macrophages, BAL cells were analyzed using single-cell RT-PCR. BW5147 cells transfected with CD8α were used as a positive control. A total of 16 macrophages from three BAL samples were chosen on the basis of their morphology. The housekeeping gene β-actin was amplified as quality control for the cDNA. RT-PCR was also performed using mouse splenic cells as a control for the primers in the PCR step. A summary of the single-cell PCR results is found in Table 8.

In Experiment #1, three cells of a BAL sample and three BW5147 cells were isolated (Figure 8). Expression of mRNA for β-actin (panel A), CD3 (panel B) and CD8 (panel C) was studied. Sample 1 of BAL cells (M1) expressed CD8 but not CD3, M2 and M3 are negative for both markers but were positive for β-actin. All three BW5147 samples (BW 4-6) were positive for β-actin, CD3 and CD8.

In Experiment #2, all samples of BW5147 and BAL cells were positive for β-actin (Figure 9). BW5147 cells (1-5) were also positive for CD3 and CD8 as shown in Figure 10 and all BAL samples (1-6) were negative for CD3 and CD8 expression as shown in Figure 11.

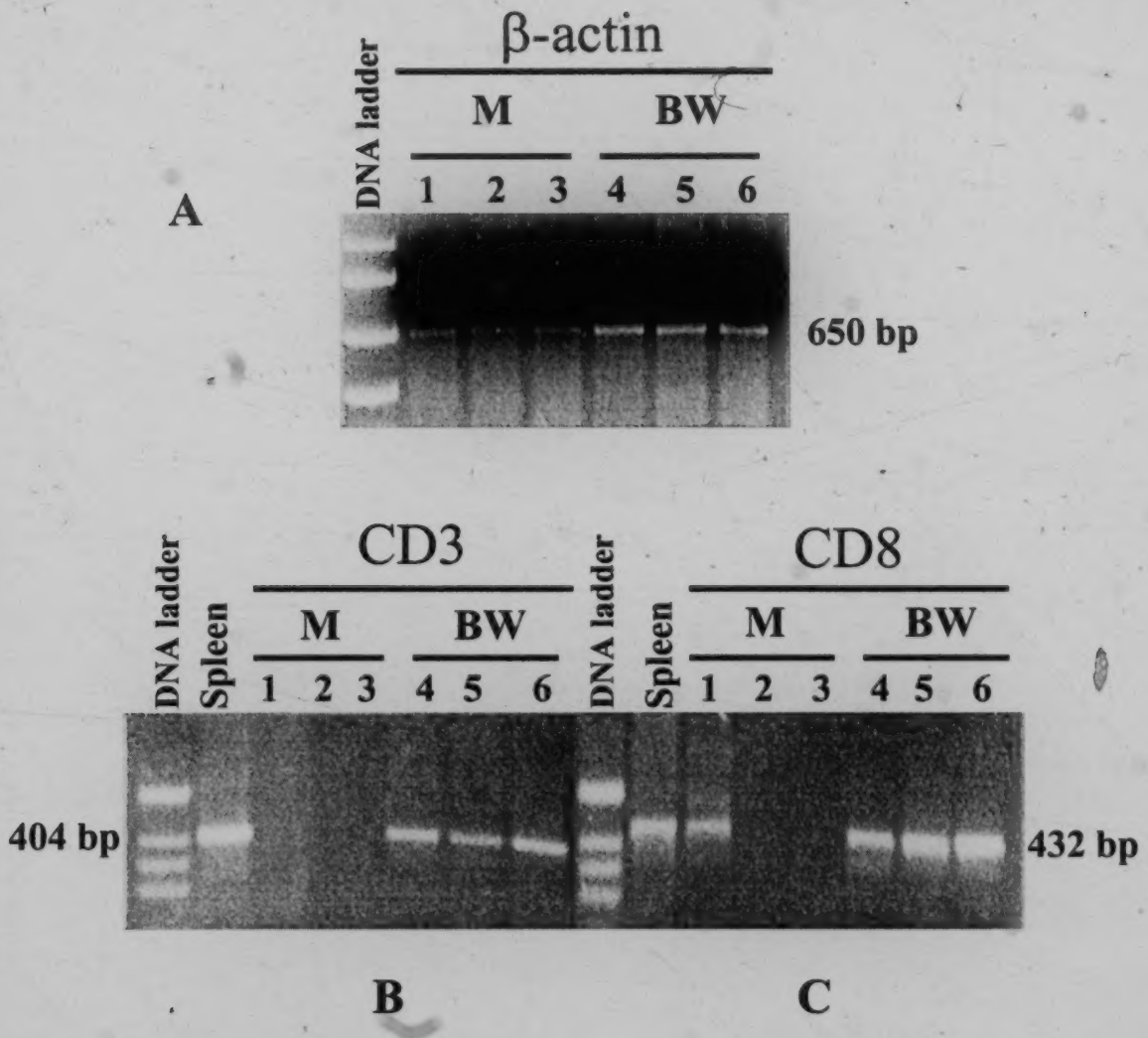


Figure 8: mRNA expression in a single cell (Experiment #1) for β-actin (650 bp; panel A), CD3 (404 bp; panel B), CD8α (432 bp; panel C) in three different macrophages (M; lanes 1-3) and BW5147 cells (BW; lanes 4-6). Splenic cells were used as a positive control.

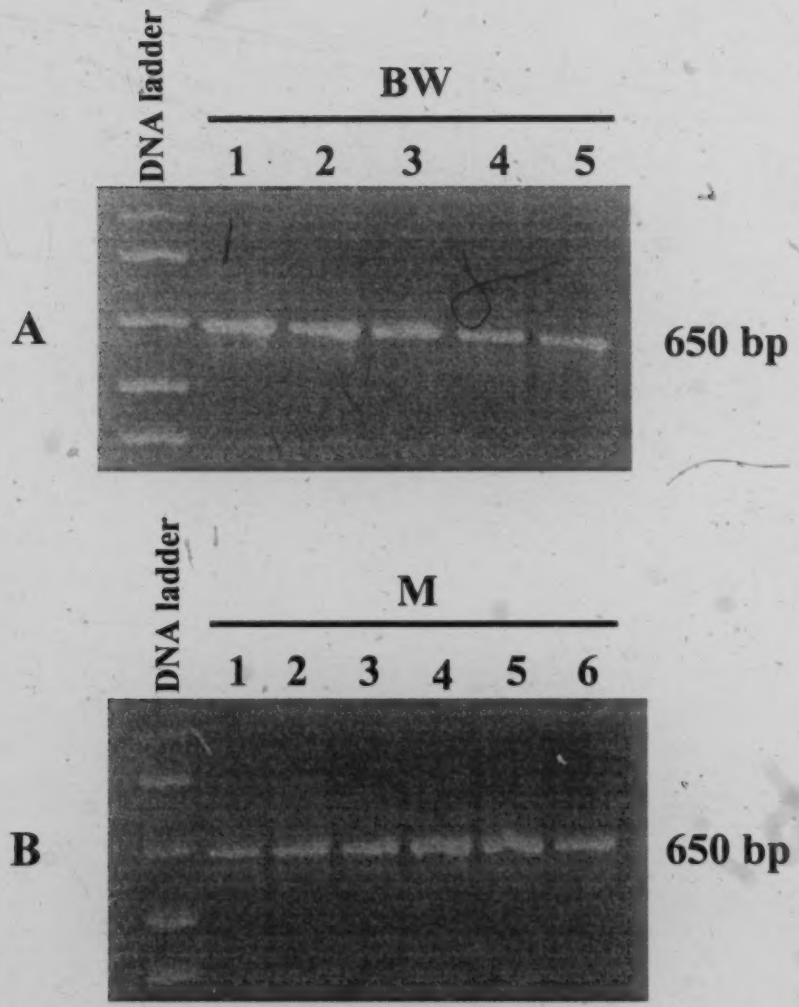


Figure 9: mRNA expression for β-actin mRNA in a single cell (Experiment #2) in five different BW5147 cells (BW; panel A, lanes 1-5) and six macrophages (M; panel B, lanes 1-6)

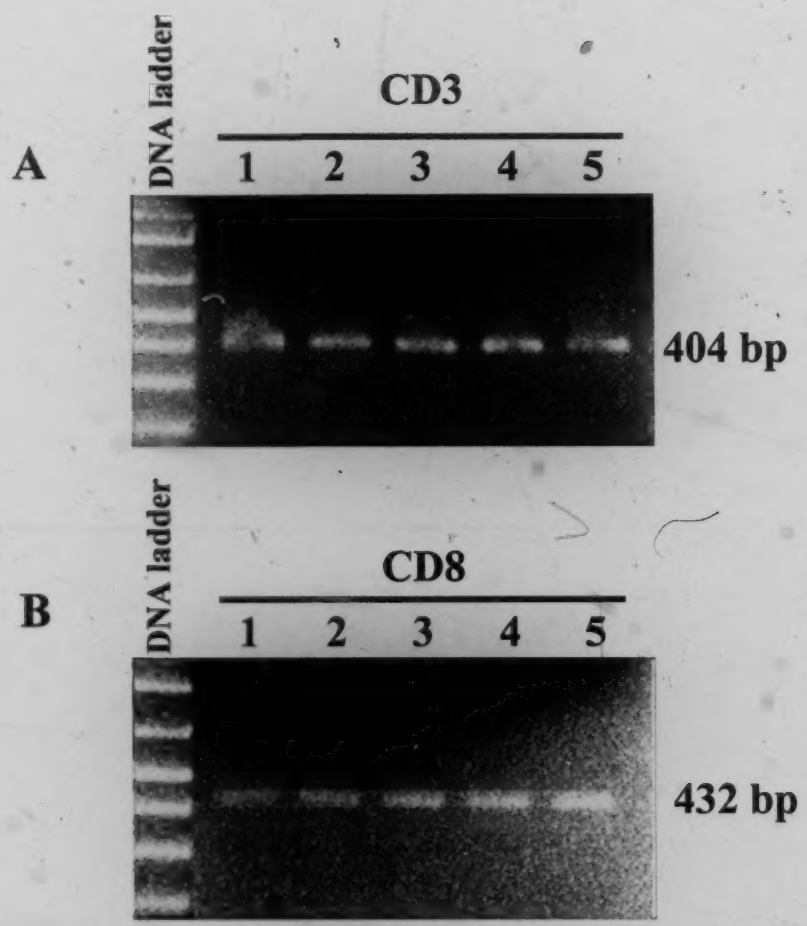


Figure 10: RT-PCR analysis in a single cell (Experiment #2). CD3 (404 bp; panel A) and CD8 (432 bp; panel B) mRNA expression in five single cells from the BW5147 cell line (lanes 1-5).

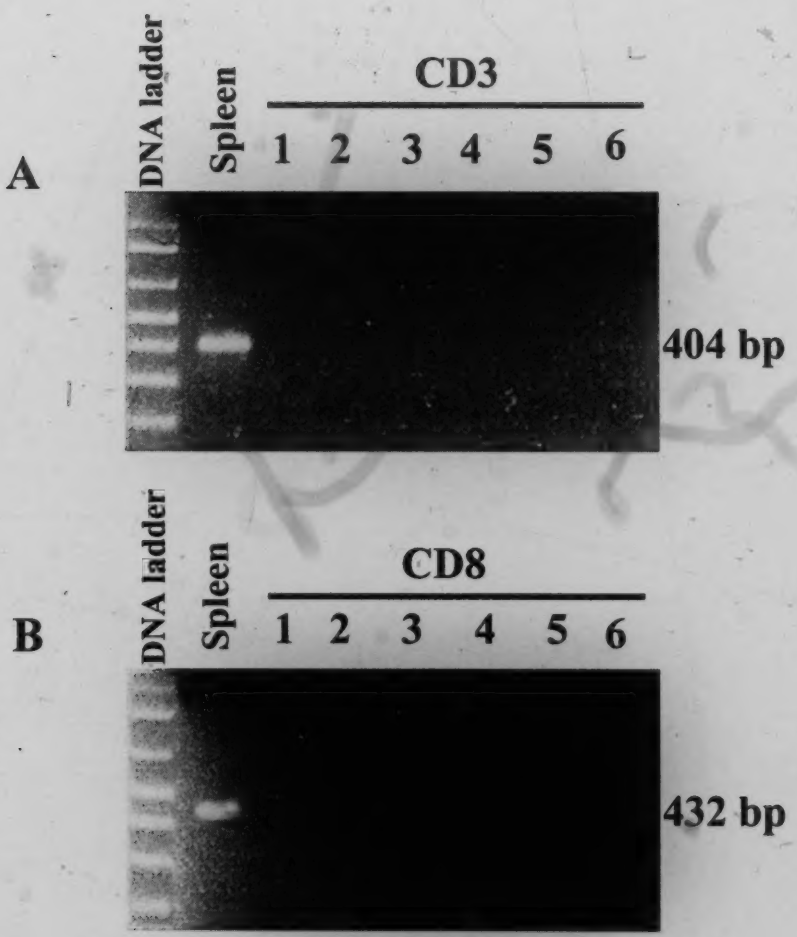


Figure 11: RT-PCR analysis in a single cell (Experiment #2). CD3 (404 bp; panel A) and CD8 (432 bp; panel B) mRNA expression in six macrophages (lanes 1-6). Splenic cells were used as a positive control.

Figure 12: RT-PCR analysis in single cells (Experiment #3). Panel A shows β-actin (650 bp), CD8 (432 bp) and CD3 (404 bp) mRNA expression in splenic cells. Panel B shows β-actin mRNA expression in seven different macrophages (650 bp; lanes 1-7).

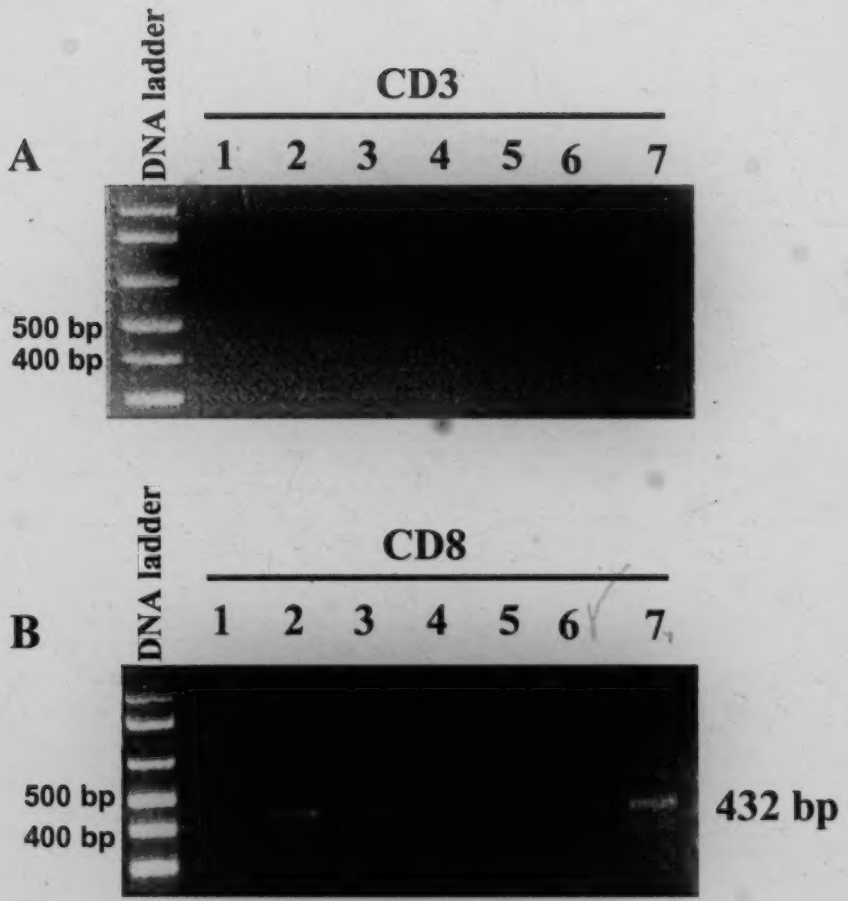


Figure 13: RT-PCR analysis in single cells (Experiment #3). mRNA expression for CD3 (404 bp; panel A) and CD8 (432 bp; panel B) in seven different macrophages (lanes 1-7). Positive controls are shown in Figure 12, panel A.

In Experiment #3, splenic cells were used as a positive control (Figure 12, panel A). All seven BAL samples were positive for β-actin as seen in Figure 12 (Panel B), although the signal in sample 2 was weak. In Figure 13 (panel A), none of the BAL samples expressed CD3, but three samples were positive for CD8 expression, i.e. sample 2, 3 and 7 (panel B).

Table 8: Single Cell PCR On BAL Cells

Total number of samples: 16

Markers	CD8+	CD8-
CD3+	0	1
CD3-	4	11

III. PCR analysis for CD8 expression in BAL cells from C57Bl/6 mice

Cells from C57Bl/6 mice lungs were used to determine whether the PCR results obtained with lavage cells from Balb/c mice were strain specific or could be generalized to other strains. The housekeeping gene β-actin was amplified as a control and was detected in all samples (Figure 14, panel A). Three samples were tested for CD3 (panel B), CD8α (primer pair 3α; panel C) and CD8β (primer pair 1β; panel D) mRNA expression. Figure 14 shows that Sample 1 was weakly positive for CD3 and positive for CD8α but negative for CD8β, Sample 2 was negative for CD3, CD8α and CD8β, and Sample 3 was negative for CD3 and CD8β but positive for CD8α. No PCR products were detected when the RT step was omitted suggesting the absence of genomic DNA in our sample.

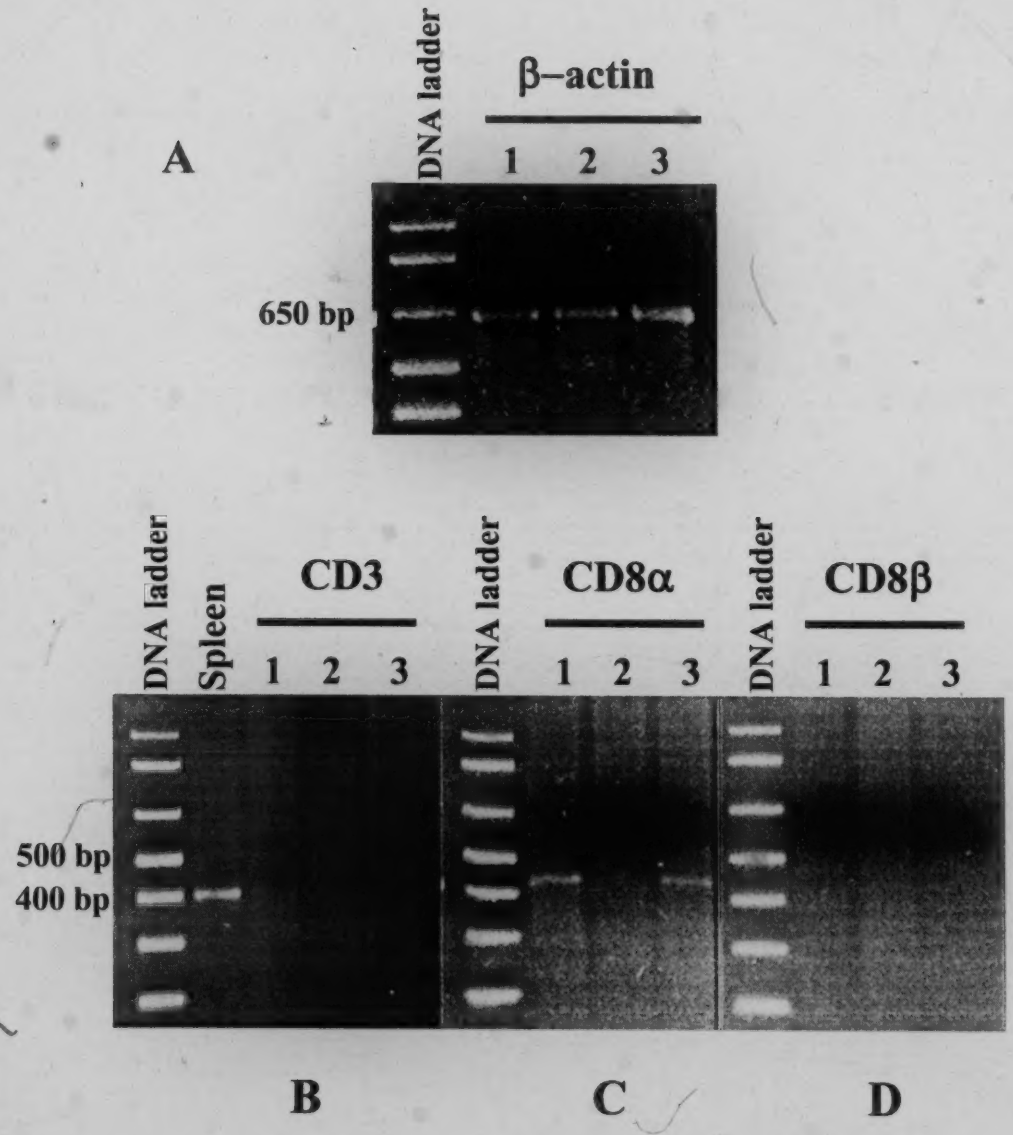


Figure 14: mRNA expression for β-actin (650 bp; panel A), CD3 (404 bp; panel B), CD8α (432 bp; panel C), CD8β (605 bp; panel D) in three different BAL samples (lanes 1-3) from C57Bl/6 mice.

IV. PCR analysis for CD8 expression in mouse macrophage cell lines

RT-PCR analysis was employed to examine the expression of CD8 mRNA in the mouse macrophage cell lines Raw264.7, MH-S, P388D1 and J774A.1. The housekeeping gene β-actin was amplified as a control and was detected in all samples (Figure 15, panel A). The mouse macrophage cell lines were negative for both CD8α (primer pair 3α; panel B) and CD8β (primer pair 1b; panel C). One preparation of RNA from MH-S and Raw 264.7 cells was positive for CD8α but it was not reproducible using the other four RNA preparations.

V. Flow cytometric analysis

Flow cytometry analysis was conducted to study the expression of CD8 protein on the surface of mouse splenic and BAL cells.

A. Rat anti-mouse CD8 Ab

CD8 protein was detected on the surface of mouse splenic cells using a range of rat anti-mouse CD8 Ab recognizing the Ig-like domain (Table 6). The 53-6.7 (n=6) and 53-5.8 Ab (n=3) labeled approximately 25% of cells from spleen tissue as $CD8\alpha^+$ (solid line) as indicated by the shift in mean fluorescence intensity (MFI) when compared to the isotype control Ab (dotted line) (Figure 16 (CD8α) and Figure 17(CD8β)). The same Ab were also tested on BAL cells (Figure 18 (CD8α; n=3) and Figure 19 (CD8β; n=3)). The MFI obtained in cells stained with anti-CD8α and

CD8β Ab (solid line) was similar to that obtained with the isotype control Ab (dotted line), suggesting that CD8α and CD8β are not expressed on mouse BAL cells. Similar results were obtained with the other rat anti-mouse Ab to CD8α and CD8β listed in Table 4.

B. Mouse anti-rat CD8 Ab

Ab recognizing rat CD8 were tested on mouse splenic cells since mouse CD8α has some homology to rat CD8α (62%) and mouse CD8β has 78% identical residues with rat CD8β. Neither OX-8 (CD8α hinge region specific; n=3) nor 341 (CD8β extracellular domain specific; n=3) could detect CD8 protein on mouse splenic cells (Figure 20). Similar results were obtained with anti-CD8α Ab G28 and R1-1085.

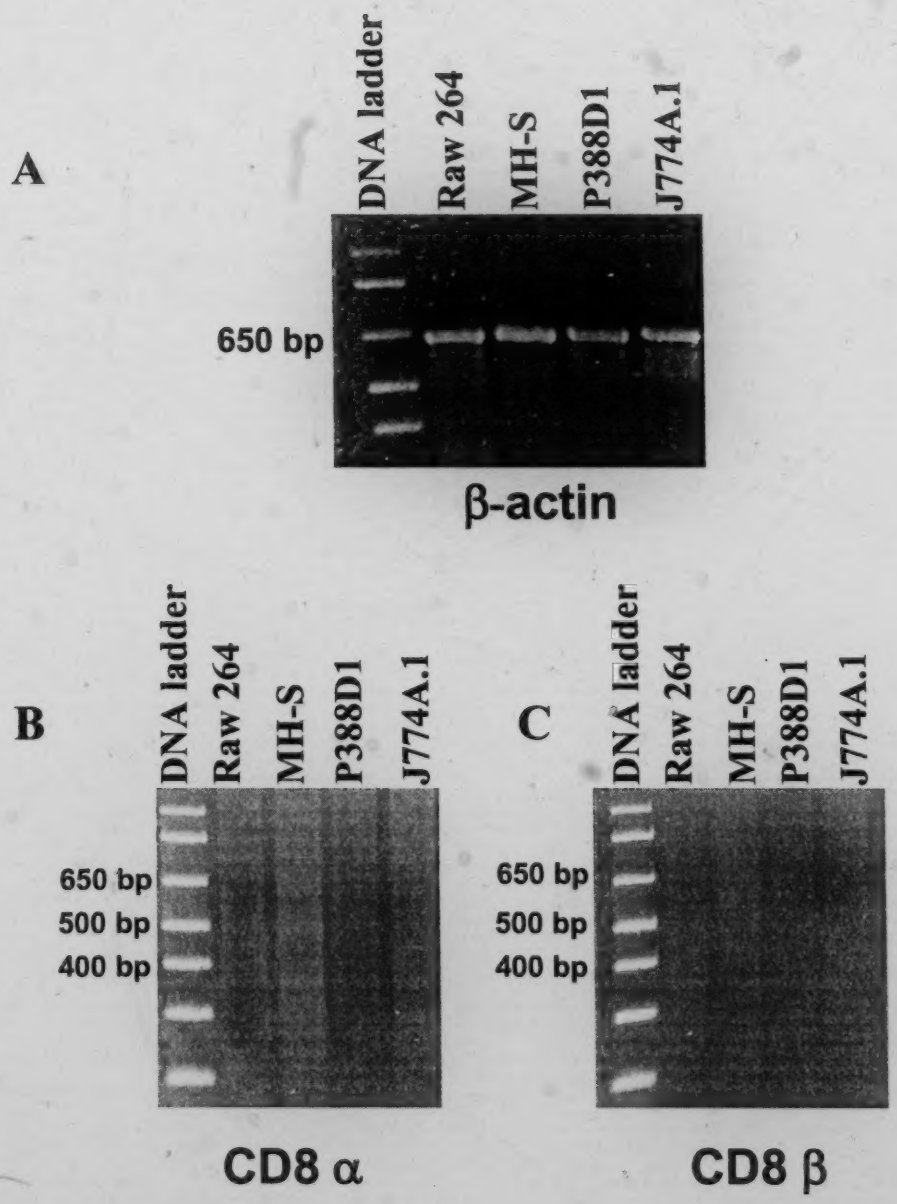


Figure 15: mRNA expression of β-actin (650 bp; panel A), CD8α (432 bp; panel B) and CD8β (605 bp; panel C) in the macrophage cell lines Raw 264.7, MH-S, P388D1 and J774A.1.

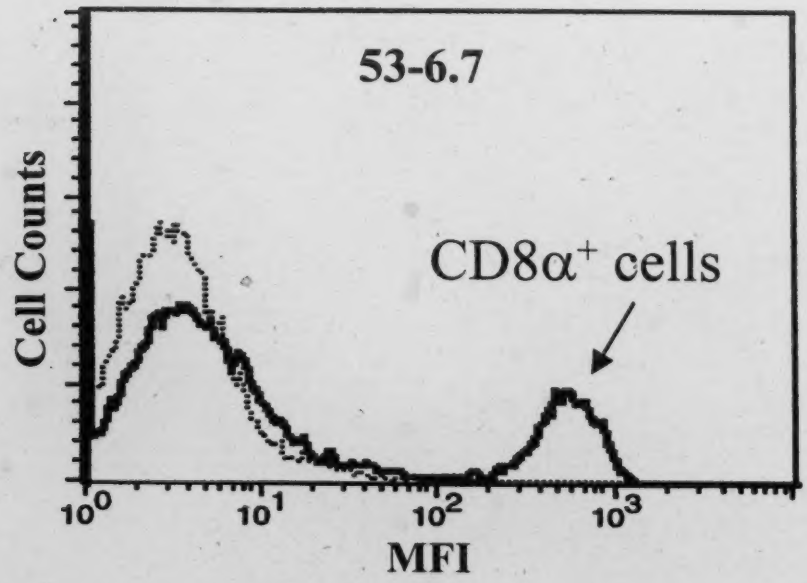


Figure 16: Detection of CD8α protein on mouse splenic cells using 53-6.7, a rat anti-mouse CD8α Ab. Dotted line: isotype control, solid line anti-CD8α Ab.

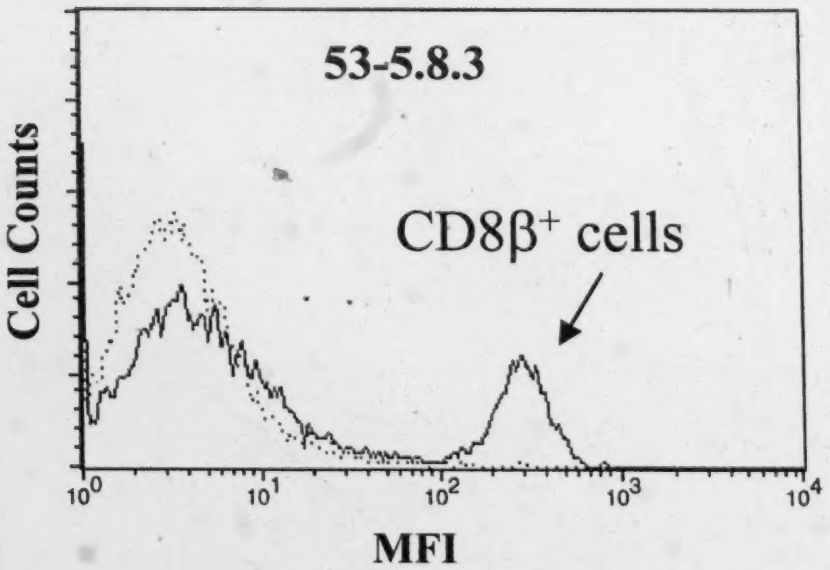


Figure 17: Detection of CD8β protein on mouse splenic cells using 53-5.8.3 Ab, a rat anti-mouse CD8β Ab. Dotted line: isotype control, solid line anti-CD8β Ab.

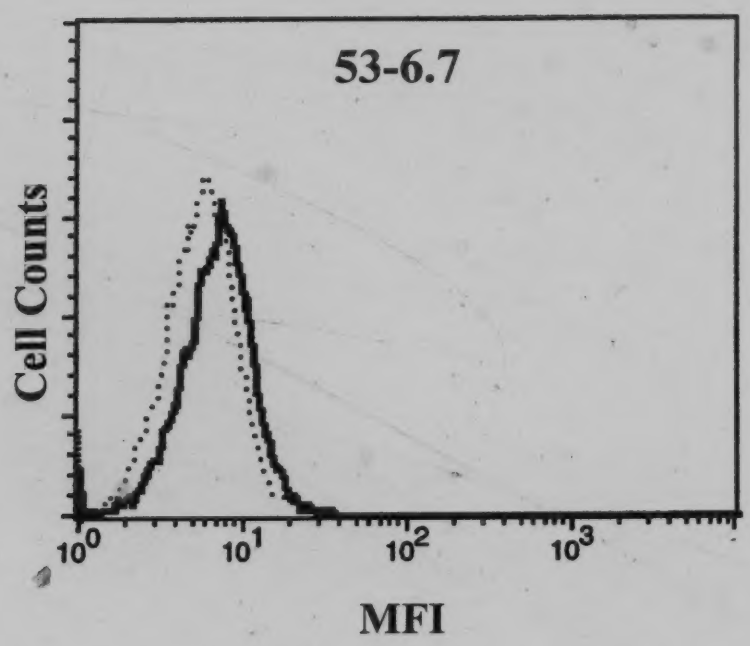


Figure 18: Detection of CD8α protein on mouse BAL cells using 53-6.7, a rat anti-mouse CD8α Ab. Dotted line: isotype control, solid line anti-CD8α Ab.

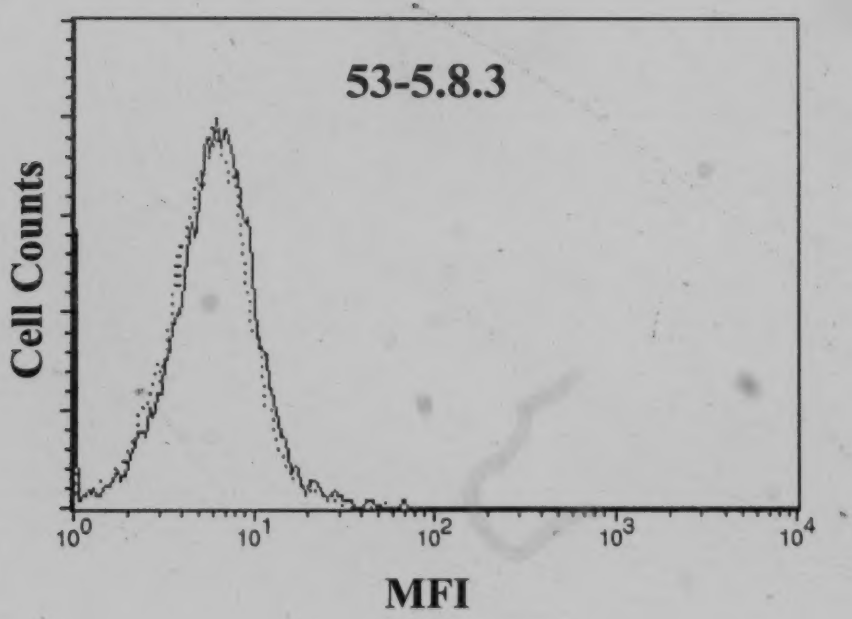


Figure 19: Detection of CD8β protein on mouse BAL cells using 53-5.8.3 Ab, a rat anti-mouse CD8β Ab. Dotted line: isotype control, solid line anti-CD8β Ab.

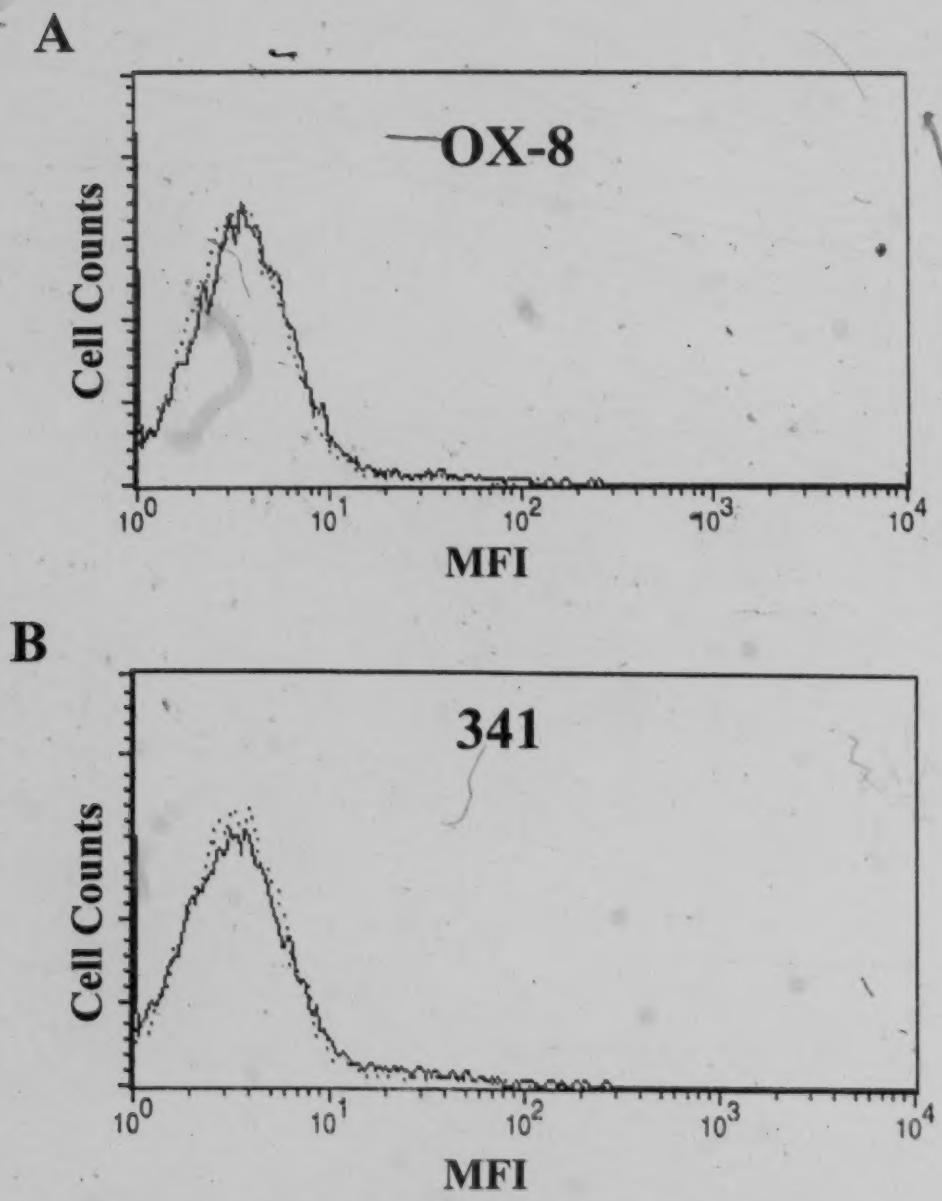


Figure 20: Studies of CD8 expression on mouse splenic cells using OX-8, a mouse anti-rat CD8α Ab (hinge region specific; panel A) or 341, a mouse anti-rat-CD8β Ab (panel B). Dotted line: isotype control Ab, solid line anti-CD8 Ab.

VI. Western blot analysis

CD8 protein expression was studied by Western blot in rat and mouse splenic cells and AM, the rat macrophage cell line NR8383 and the mouse macrophage cell lines Raw 264.7 and MH-S. For rat and mouse AM, and NR8383, 60 μg total protein was loaded in each well. For rat and mouse splenic, Raw 264.7 and MH-S, 25 μg, 35 μg, 50 μg, 50 μg of total protein, respectively, were loaded on the gel. A list of the Ab used is provided in Table 4 (Materials and methods section).

A. Rat anti-mouse CD8 Ab

Most commercially available rat anti-mouse CD8 Ab recognize cell surface CD8. These Ab were used to study the presence of denatured CD8 in cell lysates of rat and mouse splenic.

Using Ab 53-6.7 a strong signal was obtained at approximately 30 kDa for rat splenic cells and a weaker signal of similar size for mouse splenic cells (Figure 21, panel B; n=3). However, the same bands were detected using the isotype control Ab (panel A). No bands were seen in the same membranes blotted with secondary Ab only as a control for nonspecific binding. The other rat anti-mouse Ab gave similar results showing bands on the isotype control blot.

B. Mouse anti-rat CD8 Ab

OX8 is the only known Ab that detects the CD8α hinge region by flow cytometry and Western blot. Due to the lack of Ab recognizing mouse CD8α hinge

region and the fact that part of rat CD8α hinge region has 77% homology to mouse CD8α hinge region, OX-8 was tested for recognition of mouse CD8 by Western blot. The following cell types were screened for CD8 expression using the OX8 Ab: rat and mouse splenic and AM, the rat AM cell line NR8383, and the mouse macrophage cell lines Raw 264.7 and MH-S. A representative experiment is shown in Figure 22 (n=6).

A band at approximately 42 kDa was detected by the isotype control Ab on rat AM and NR8383 (panel A), suggesting that these bands resulted from nonspecific binding. The band of approximately 42 kDa was also observed for most cell types except for the mouse cell lines (panel B). The band intensity was weak for mouse splenic cells and got increasingly stronger for rat AM, rat splenic cells and NR8383. Higher molecular weight bands were detected at ~ 48 and ~ 54 kDa that have previously been described for OX8 staining (17).

The anti-rat CD8 Ab 341 (CD8β), G28 (CD8α) and R1-1085 (CD8α) detect CD8 on T cells by flow cytometry. However, these Ab, were not able to detect rat or mouse CD8 by Western blot on splenic cells.

A washing step with 3 M urea was used on membranes blotted with OX8 Ab to reduce low affinity binding of proteins (Figure 23; n=3). The blot incubated with the isotype control showed two weak bands at approximately 65 kDa for NR8383 and mouse AM, which were also present on the membrane blotted with OX8, suggesting nonspecific binding of the Ab (panel A). Following the urea incubation, rat splenic cells showed a doublet band of approximately 35-38 kDa when incubated with OX8 (panel B).

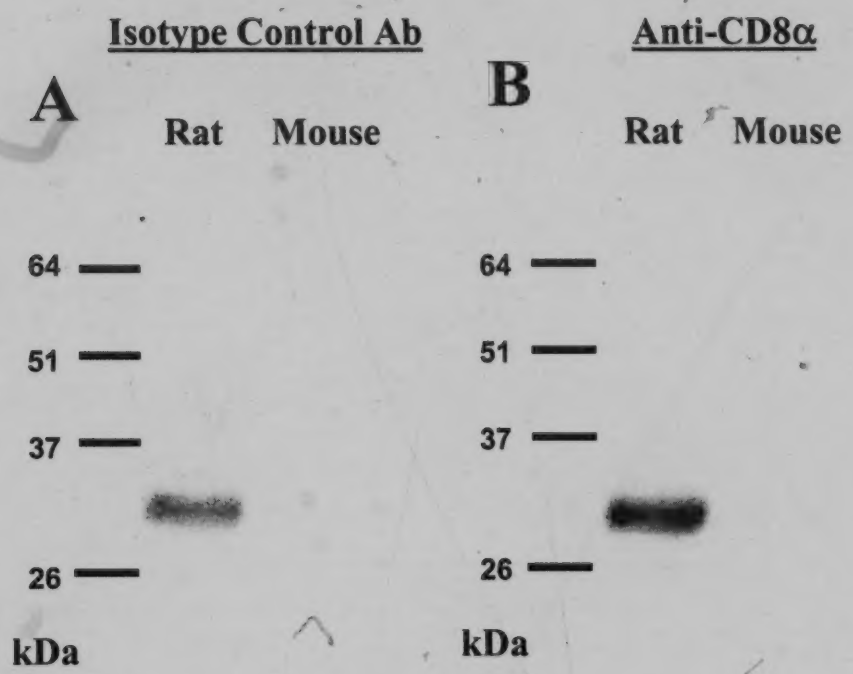


Figure 21: Western blot analysis of rat mouse splenic cells showing nonspecific binding to a band at 30 kDa. Membranes were blotted with rat IgG (isotype control; panel A) or rat anti-mouse CD8α mAb, 53-6.7 (Ig-like domain specific mAb; panel B).

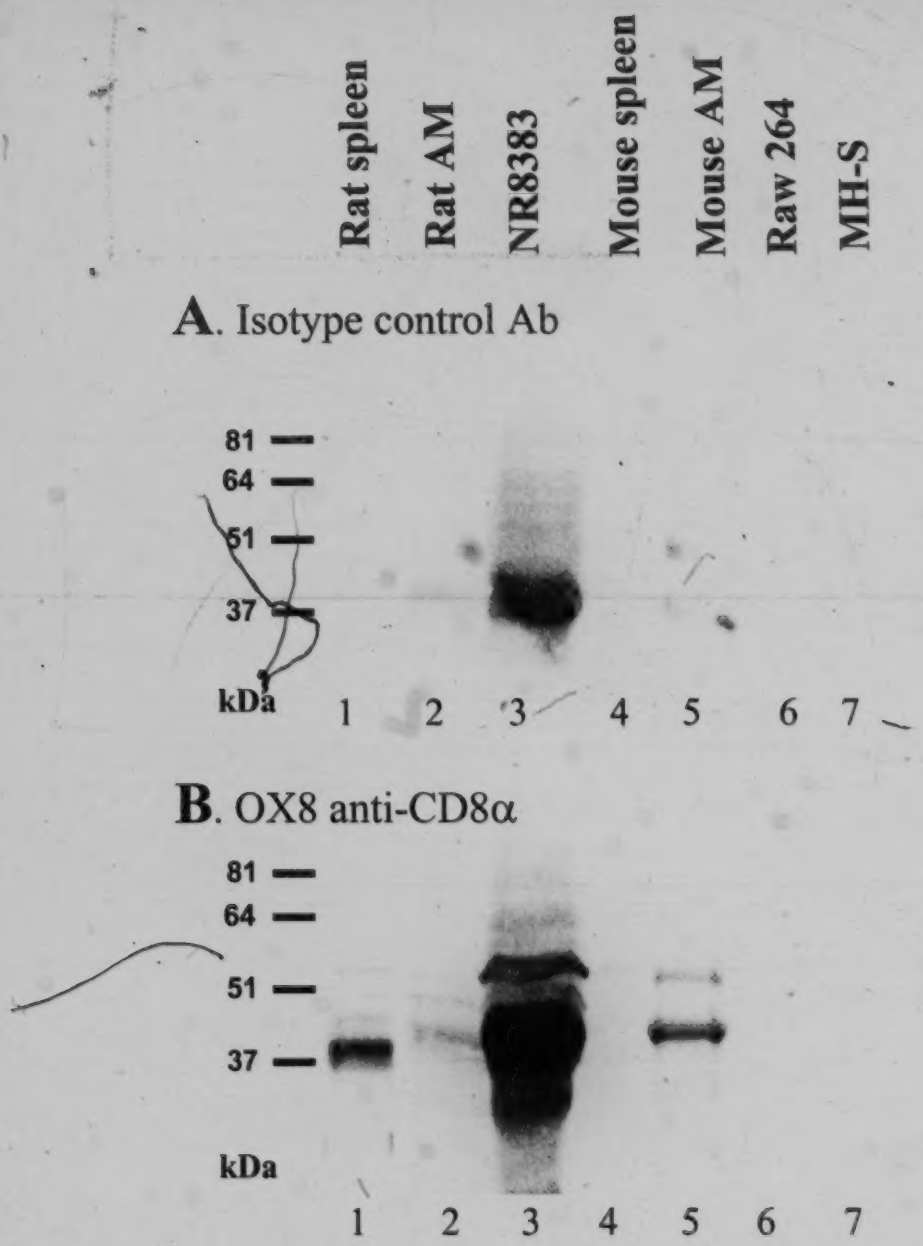


Figure 22: Western blot analysis of rat splenic cells (lane 1), rat BAL cells (lane 2), NR8383 (lane 3), mouse splenic cells (lane 4), mouse BAL cells (lane 5), Raw 264 (lane 6) and MH-S (lane 7) for the expression of CD8α. Membranes were blotted with mouse IgG_1 (isotype control; panel A) or mouse anti-CD8α mAb, OX-8 (hinge region specific; panel B).

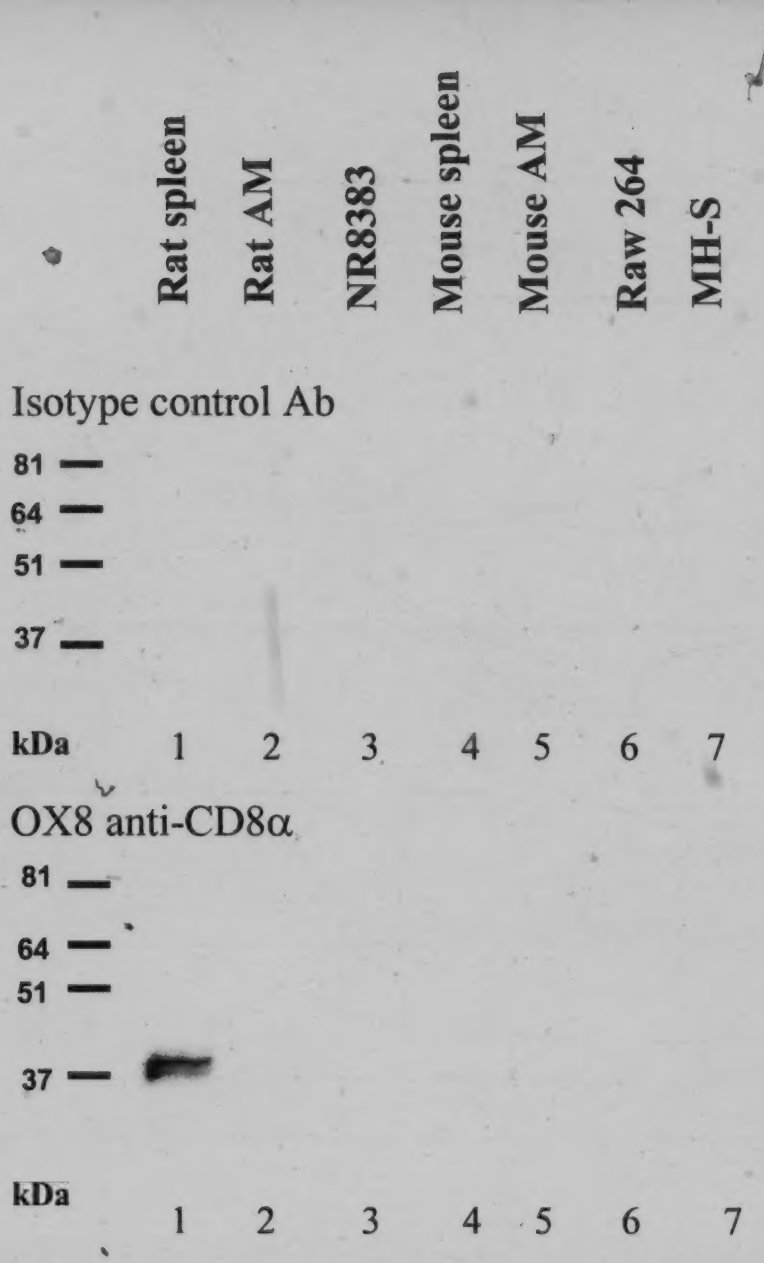


Figure 23: Western blot analysis of rat splenic cells (lane 1), rat BAL cells (lane 2), NR8383 (lane 3), mouse splenic cells (lane 4), mouse BAL cells (lane 5), Raw 264 (lane 6) and MH-S (lane 7) for the expression of CD8 after treatment of the membranes with 3 M urea. Membranes were blotted with mouse IgG1 (isotype control; panel A) or mouse anti-CD8α mAb, OX-8 (hinge region specific; panel B).

C. D-9, a monoclonal mouse anti-mouse, rat and human CD8α Ab

To investigate the presence of CD8 protein in mouse splenic and BAL cells, a mouse monoclonal Ab raised against a peptide sequence present in the extracellular domain of CD8α was tested by Western blot. Rat and mouse splenic cells and AM, the rat AM cell line NR8383, the mouse macrophage cell lines Raw 264.7 and MH-S were used (representative experiment shown in Figure 24; n=4).

The isotype control Ab detected a band at approximately 42 kDa for rat and mouse AM as well as for NR8383 and a band higher than 51 kDa for mouse AM (panel A). No bands were detected by the isotype control Ab for rat and mouse splenic cells and the mouse cell lines Raw 264.7 and MH-S. D-9 also detected a band at ~ 42 kDa on rat splenic cells and AM, NR8383 and mouse AM (panel B). Only the band from rat splenic cells was not detected by the isotype control Ab. Another band of ~ 50 kDa was detected in rat and mouse AM, mouse splenic cells and MH-S cells. With the exception of mouse AM, this band was not detected by the isotype control Ab.

An extra washing step with 3 M urea was performed on membranes blotted with D-9 Ab (Figure 25; n=2). The isotype control Ab did not detect any bands (panel A). A band at approximately 42 kDa was observed in rat cells but not for mouse cells (panel B). A band at ~ 50 and ~ 57 kDa was also present in NR8383 and mouse splenic cells respectively.

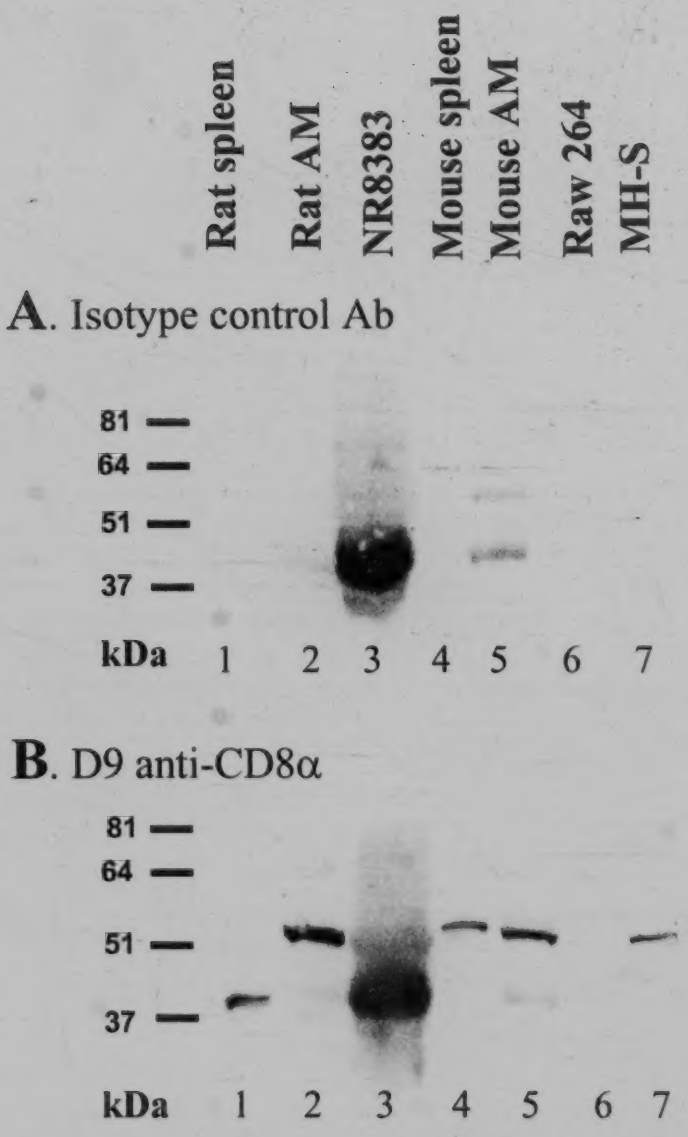


Figure 24: Western blot analysis of rat splenic cells (lane 1), rat BAL cells (lane 2), NR8383 (lane 3), mouse splenic cells (lane 4), mouse BAL cells (lane 5), Raw 264 (lane 6) and MH-S (lane 7) for the expression of CD8. Membranes were blotted with mouse IgG_2 (isotype control; panel A) or mouse anti-CD8α mAb, D9 (extracellular domain specific; panel B).

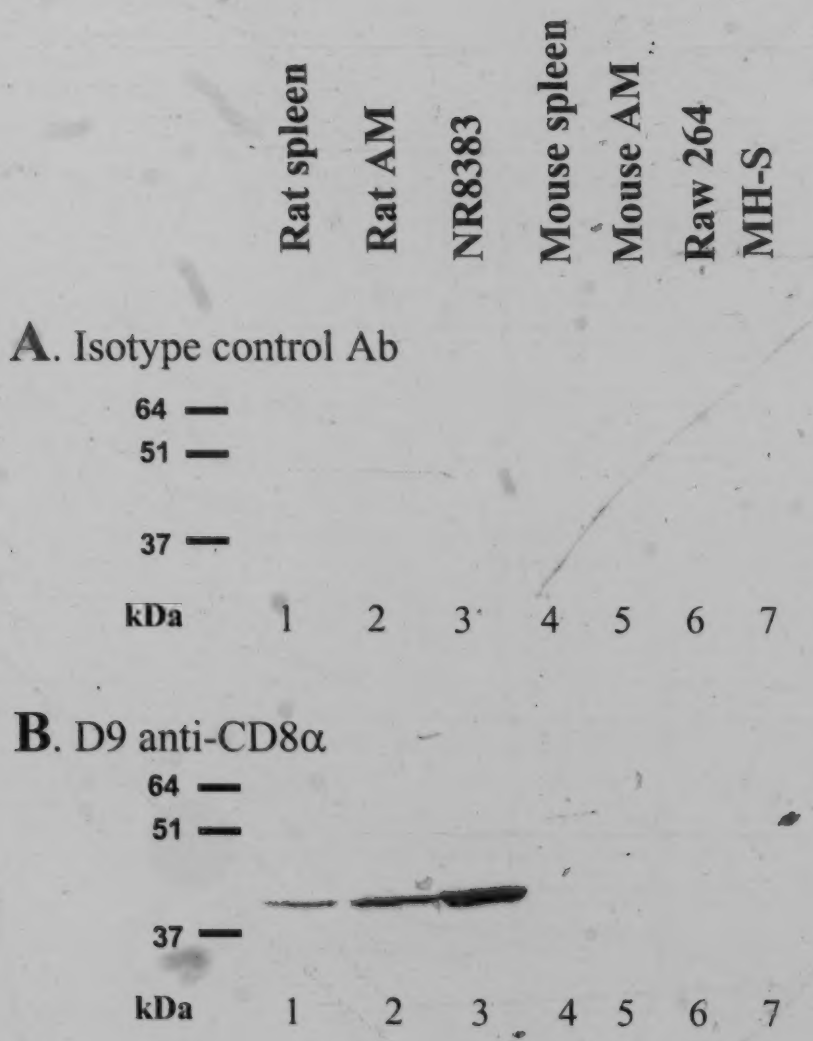


Figure 25: Western blot analysis of rat splenic cells (lane 1), rat BAL cells (lane 2), NR8383 (lane 3), mouse splenic cells (lane 4), mouse BAL cells (lane 5), Raw 264 (lane 6) and MH-S (lane 7) for the expression of CD8 after treatment of the membranes with 3 M urea. Membranes were blotted with mouse IgG_2 (isotype control; panel A) or mouse anti-CD8α mAb, D9 (extracellular domain specific; panel B).

D. H-160, a polyclonal rabbit anti-mouse, rat and human CD8α Ab

A polyclonal Ab raised against a peptide sequence contained in the extracellular domain of CD8α was used to detect CD8 in rat and mouse splenic cells, a rat AM cell line NR8383, and the mouse macrophage cell lines Raw 264.7 and MH-S. A representative experiment is shown in Figure 26 (n=5).

The membranes blotted with the isotype control Ab showed weak bands higher than 37 kDa for all cell types as well as a strong band at ~ 29 kDa for NR8383 (panel A). Blotting with H-160 resulted in the detection of stronger bands in each sample at ~ 26 kDa and at ~ 39, ~ 55 and ~ 69 kDa (panel B). A strong band at approximately 79 kDa was also detected for NR8383 and Raw 264.7. Bands at ~ 33 kDa were also present for NR8383, mouse splenic and Raw 264.7 cells.

Following an extra washing step with 3 M urea, no bands were detected by the isotype control Ab (Figure 27, panel A; n=2). Bands at ~ 55 and ~ 65 kDa for all the cell types and at approximately 26 and ~ 33 kDa for NR8383, mouse splenic and Raw 264.7 cells could still be observed after the membrane was blotted with H-160 (panel B). A band at approximately 39 kDa was observed for mouse splenic cells and Raw 264.7 and bands at ~ 73 and ~ 79 kDa were detected for Raw 264.7.

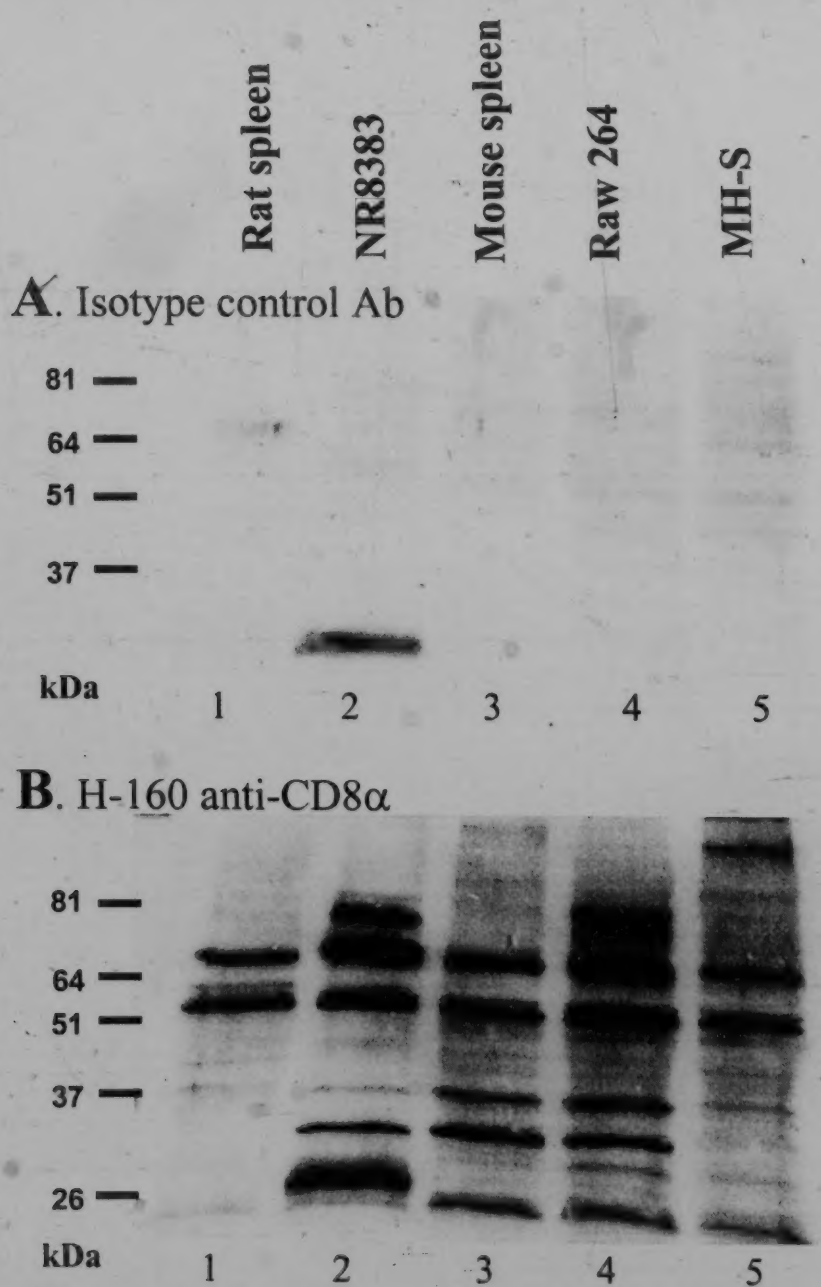


Figure 26: Western blot analysis of rat splenic cells (lane 1), NR8383 (lane 2), mouse splenic cells (lane 3), Raw 264 (lane 4) and MH-S (lane 5) for the expression of CD8. Membrane were blotted with rabbit IgGs (isotype control; panel A) or polyclonal rabbit anti-mouse CD8α Ab, H-160 (extracellular domain specific; panel B).

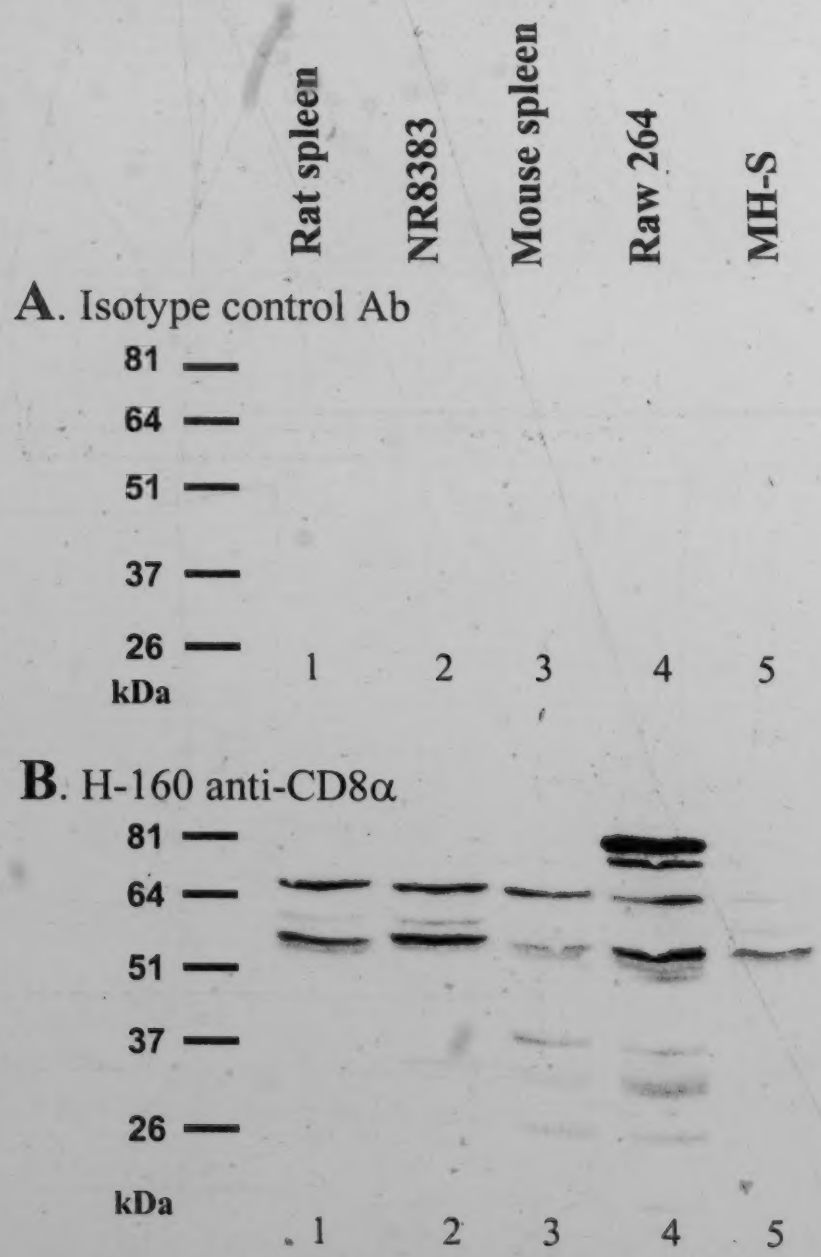


Figure 27: Western blot analysis of rat splenic cells (lane 1), NR8383 (lane 2), mouse splenic cells (lane 3), Raw 264 (lane 4) and MH-S (lane 5) for the expression of CD8 after treatment of the membranes with 3 M urea. Membrane were blotted with rabbit IgGs (isotype control; panel A) or polyclonal rabbit-anti-mouse CD8α Ab, H-160 (extracellular domain specific; panel B).

CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

I. DISCUSSION

Our lab identified a novel CD8 molecule on rat macrophages. My aim was to extend studies of rat macrophage CD8 to a mouse model. It was anticipated that this would facilitate the study of CD8 functions on AM. Mouse BAL cells were shown to express CD8 mRNA. Moreover, CD8α mRNA expression was detected in a macrophage-looking subpopulation of BAL cells using single-cell RT-PCR. Unfortunately, CD8 protein in or on mouse AM was not detected. These results contrast with rat and human macrophage studies that showed CD8 expression on AM from both species. Some of the potential reasons for these differences are discussed below, and suggestions are made for further investigations.

BAL

A limitation of the BAL technique is the possibility that it selects macrophages that were non-adherent in vivo. Lavage with physiologic salt solutions may select against strongly adherent macrophages, whose morphological and functional properties may be dissimilar from those of less adherent cells. Thus, EDTA was added to our buffer to lavage the lungs, anticipating that this would increase the recovery of adherent cells, leading to recovery of a more representative macrophage population. Despite this approach, no $CD8^+$ AM were identified by flow cytometry.

mRNA expression

To expand our knowledge about the expression of CD8 among cell types and species, CD8 mRNA expression in mouse BAL cells was investigated. Primers pairs were designed to amplify various regions of the CD8 molecule and optimized using splenic cells (Figure 3). All primer pairs led to the amplification of CD8 in BAL cells (Figure 5). However, the signal obtained with primer pair 5α was weaker than that obtained with other primer pairs. Although the primer pair 5α also gave the weakest signal for CD8 in T cells, its product was not consistently detected in BAL cells. This suggested that the mRNA sequence for CD8 in BAL cells could be different from the one in T cells for the hinge region, since the sequence recognized by primer pair 5α is part of the hinge region. However, sequencing the products obtained with the primer pair 5α showed that the PCR product from BAL cells shared 97% identity with T cell CD8. Moreover, sequencing of the products obtained with primer pair 1α, also determined that the sequence amplified in BAL cells was 98% identical to T cell CD8. These 2 to 3% differences in the sequences, as compared to published T cell CD8α sequences, were largely due to weak signals that prevented identification of bases. Conclusions cannot be drawn about the additional nucleotides observed in the sequences of PCR products from primer pairs 3α and 5α because of insufficient results. The observations could be attributed to technical error if the signal was weak, or represent an alternative sequence for CD8 in mouse macrophages. The latter could lead to a frame shift and thus, eventually to an alteration in protein translation, explaining the difficulty in identifying the surface molecule.

The macrophage population obtained by BAL contained approximately 2% cells with morphological characteristics of lymphocytes. Similar numbers were found in other rodent systems. In normal mice, a lymphocyte population of 3.2% and 1.1% of neutrophils were obtained which is consistent with our findings (126). In Brown Norway rats, the lymphocyte population is higher than what was observed in our studies and represents approximately 7% of total BAL cells, 5.4 % being T lymphocytes (2% being CD8+), 0.9% B lymphocytes and 0.7% NK cells (127). Given that PCR is an extremely sensitive technique and would most likely be able to detect mRNA in 2% contaminating $CD8^+$ T lymphocytes, co-expression of CD3 was also looked at, a T cell marker (Figure 7). It was found that 53% of the samples were positive for CD8 expression and also positive for CD3, suggesting that the CD8 expression could be attributed to a population of T-cells in BAL (Table 5). Only 17% of the samples tested were positive for CD8, but negative for the T cell marker CD3 suggesting that CD8 expression in those samples could be attributed to the presence of other cell types expressing CD8, possibly AM.

The possibility cannot be excluded that other cell types found in the lungs aside from macrophages could be expressing CD8. Some such cell types previously identified in BAL fluids include DC, B lymphocytes and NK cells (127,128). Using standard techniques, DC are rarely found in BAL fluid, but CD8α is expressed by mouse thymic and splenic DC and amplification of DC mRNA could account for our PCR results (50,53,129). To our knowledge, there has been no report on CD8 expression on mouse B cells, although CD8 has been identified on $CD19^+$ human B cells. Mouse NK cells unlike rat and human NK cells are CD8 negative (47,48). Neutrophils have also been identified

in some of the samples, but represent less than 1% of total BAL cells. Moreover, no reports have shown CD8 expression on this cell type, making them an unlikely source of our positive PCR results in BAL cells. Importantly, none of these cell types are known to be positive for CD8β and, therefore, their presence as contaminating cells does not support our PCR results obtained in BAL samples for CD8β mRNA expression.

It is important to mention that macrophages and DC are heterogeneous for their surface markers, depending on their microenvironment. Thus, one could speculate that DC are a prime candidate for a cell expressing CD8α in the lungs besides macrophages. However, whether CD8α mRNA expression occurs in the other cell types, including NK and B cells and neutrophils, must be addressed in future studies. A technique such as *in situ* RT-PCR would help answer these questions, however this technique was unsuccessful in my hands (data not shown).

Given concerns that our RT-PCR results could be due to the impurity of AM populations, single-cell PCR was used to analyze CD8α mRNA expression from macrophage-looking cells in BAL samples. These single cells were also tested for CD3 expression. Eleven cells out of 16 were negative for both CD8 and CD3, but 4/16 cells expressed CD8 but not CD3. This suggests that the cells positive for CD8 are not T cells.

The single-cell RT-PCR study would have been more conclusive if a macrophage marker was also tested along with the T cell marker CD3. The use of F4/80, which is a macrophage-specific glycoprotein, could be considered for future studies. Markers for other cell types such as CD11c and CD209 for DC, CD19 for B lymphocytes, CD56 for NK cells and elastase for neutrophils could also be valuable in determining which cell type is positive for CD8α mRNA. However, single-cell RT-PCR presents some

limitations in the amount of cDNA obtained from a single cell. In this study, 1/3 of the total cDNA obtained per cell has been used for each amplification by PCR. Three markers were studied, β-actin, CD8 and CD3. Having a total of 20 μL of cDNA per RT reaction, no additional PCR reactions could be performed on the cDNA obtained. It is possible that further optimization of this technique would allow one to use smaller amounts of cDNA and therefore a greater number of experiments could be performed on one sample. The number of individual cells studied should also be increased to provide more confidence in the results.

A number of reports have described the isolation of different populations of pulmonary macrophages. Characterization of AM and IM has identified morphological and functional differences between these two populations of lung macrophages (96,130,131). The main morphological characteristics of macrophages in the interstitium that distinguish them from AM include their smaller size, smoother cell surface, and limited number of lysosomes (96,131). Overall, IM resemble monocytes more than AM do. They are functionally distinct from AM because IM are less phagocytic and adhere less to vitronectin and fibronectin than AM. IM also seem to express less CD45 and transferrin receptor, but more CD54 than AM (96). However, IM are not found in BAL fluid and are obtained by disruption of lung tissue using collagenase. The latter method presents some limitations since a high number of AM are also found in lung tissue preparations and there is currently no method to separate AM and IM.

There are studies that describe subpopulations of AM. These subpopulations have been characterized mostly in human and rat using discontinuous albumin gradients or continuous Percoll gradients (132-135). These reports describe four to five fractions of

cells that have different densities. The density of cells has been related to their maturation. Fractions with the lowest density contain bigger cells with less peroxidase activity than cells found in the densest fractions. The smallest cells found in the densest fractions also have fewer vacuoles, but more lysosomes. All of these morphological characteristics led to the assumption that the generation of these subpopulations of AM is a reflection of different stages of maturation for AM, cells becoming less dense as they mature.

Studies of the functions of AM also identified functional heterogeneity among subpopulations. The attachment of AM to opsonized erythrocytes determined that fractions in the middle of the density gradients were most efficient at binding Ab-coated erythrocytes (132). They responded to a lower concentration of Ab than other fractions. The ingestion of latex beads has also been studied (133). Cells of lower density (older) were better at ingesting beads than cells with higher density. When different fractions were incubated with whole lymph nodes cells, suppression of ConA activated-lymphocyte proliferation was observed by the middle fractions, but not by the cells with high density (133). Thus there are functional differences among AM subpopulations that can be attributed to changes in maturation or to external stimuli.

It is possible that CD8 expression could be part of the characteristics that distinguish AM at different stages of maturation, or AM responding to a particular stimulus. Knowing that subpopulations of AM with different morphological and functional properties are recovered by BAL, it is attractive to postulate that one or more of these subpopulations could express CD8.

To establish if our findings in Balb/c mice were applicable to other mouse strains, RT-PCR analysis was also performed on three BAL samples from C57Bl/6 mice (Figure 14). One sample was positive for both CD3 and CD8, one sample was negative for both markers and one sample was positive for CD8 only. These results are consistent with previous experiments on Balb/c mice BAL cells. Interestingly, the two samples positive for CD8 were taken from mice ordered on the same date but at different ages. The sample positive for CD8 only came from older mice suggesting that CD8 expression could be upregulated as the cell populations in the lungs of older animals change and mature. This postulate should be rigorously tested.

Protein expression

The anti-rat CD8α Ab, OX8, is unique in that it recognizes the hinge region of CD8α. All the commercially available Ab to human and mouse CD8α where the epitope-specificity is know identify epitopes within the Ig-like ligand-binding domain. This domain is believed to be different on rat macrophages compared to T cells. Therefore, several Ab were tested for the recognition of mouse CD8α, trying to identify at least one that would bind to CD8 on macrophages.

Using flow cytometry, rat anti-mouse CD8 Ab listed in Table 4 were optimized using mouse splenic cells and showed that 25% of these cells were positive for CD8. Unfortunately, none of these reagents detected CD8 on mouse BAL cells. This means that CD8 is either not expressed on BAL cells or that the CD8 molecule expressed on these cells has a different Ig-like domain, as suggested by studies in rat macrophages (5).

Mouse anti-rat CD8 Ab were also tested for recognition of mouse CD8. The purpose was to determine if OX8 would cross-react with mouse CD8, because the CD8α hinge domain has 84% identical residues between rat and mouse. Unfortunately, this Ab was unable to detect CD8 on mouse splenic cells and therefore, no further studies were conducted with mouse macrophages using OX8. Interestingly, OX8 identifies a neuraminidase-sensitive epitope on rat CD8.

Due to the lack of an Ab recognizing mouse CD8α hinge region, monoclonal Ab were developed against a peptide sequence for mouse CD8α hinge region by Dr. Pearson of University of Victoria. Ninety different hybridoma supernatants specific for the immunizing peptides by ELISA were screened by flow cytometry for CD8 on a cell line transfected or not with CD8α. The results obtained with both cell lines were similar, indicating that none of the supernatants detected CD8 on the surface of the cells. Since the Ab were raised to a linear peptide they are more likely to recognize a denatured form of CD8 and it would have been valuable to test the supernatants by Western blot. Unfortunately, little volume of the supernatants was left for further experiments. The hybridomas were lost following the breakdown of the freezer they were stored in at University of Victoria.

To evaluate the possibility that commercial Ab tested by flow cytometry could detect denatured CD8, Western blot analysis was used. Some of the rat anti-mouse CD8 Ab such as 2.43, 53-6.7.2 and 53-5.8.3, have previously been used for immunoprecipitation studies but are not known to work for Western blot analysis. None of these Ab recognized mouse CD8α or CD8β on splenic cells by Western blot.

Mouse anti-rat CD8 Ab were also tested by Western blot for potential cross-reactivity with mouse CD8. These Ab did not recognize mouse CD8α or CD8β on splenic cells and AM. The mAb D9 recognized rat but not mouse CD8α and the polyclonal Ab H-160 recognized more than one protein suggesting nonspecificity. In summary, despite all our efforts no Ab were found that detected CD8 on mouse splenic cells or AM by Western blot.

Expression of CD8 on multiple cell types

Our knowledge of CD8 has evolved markedly over the past years. The description of pattern of CD8 expression on multiple cell types, only recently recognized, and the identification of new ligands for CD8 have stimulated investigation into potential novel roles for CD8. Because CD8 expression is more widespread than previously thought, CD8 may have several important roles in regulation of cell functions and/or cell interactions. Moreover, a CD8 molecule, which appears to be different from the one on T cells, has been identified on rat AM, PM and PMC. The epitope recognized by G28 Ab in the Ig-like region of CD8α is detected on T cells, but not on rat AM, PM and PMC suggesting that there is an alteration of the Ig-like domain of CD8α in these cells.

These observations are consistent with preliminary results obtained in our lab using flow cytometric analysis of human monocytes, AM and a human monocyte-like cell line, THP-1. Human monocytes and macrophages express a form of CD8α that does not bind certain anti-CD8α Ab that recognize T cell CD8. D9 and 32-M4 Ab detected CD8α on human monocytes and macrophages, while LT8 did not. These results support

our previous data for rat CD8 on macrophages and MC. This data with human and rat cells suggest that the results obtained for mouse macrophages using flow cytometry could be due to the lack of an Ab recognizing epitopes outside the Ig-like domain of CD8α.

Proposed roles of CD8 on cells other than T cells

One of the suggested roles for the novel CD8 described on rat macrophages and MC is as a signaling molecule (5). Cross-linking of CD8 on rat macrophages and MC stimulated the release of NO, TNF and IL-1β suggesting an important role of CD8 in mediator secretion (40,94). CD8 might also be involved in leukocyte differentiation and maturation. For example, CD8 is upregulated in certain diseases such as acquired immunodeficiency syndrome, systemic lupus erythematosus and rheumatoid arthritis, probably in response to a change in the growth factors present in the environment (60,61,89). CD8 expression on immune cells could also be upregulated to facilitate release of soluble CD8 that might stimulate or suppress cells in a paracrine fashion (27,29,30). Expression of CD8 can be triggered on CD4+ T lymphocytes using mitogens such as PHA and ConA, suggesting that CD8 expression could be upregulated as a mechanism to dampen progression of the immune response (28,58,59). To support this theory, it has also been observed that glucocorticoids, which lead to immunosuppression, induce CD8α expression on activated rat $CD4^+$ T lymphocytes (57). A role of immunosuppression for CD8 has been more extensively described for CD8 expressed on DC. It has been proposed that CD8 might be involved in triggering thymocyte apoptosis by interacting with the α3 domain of MHC class I on thymocytes (88).

Monocytes may alter expression of CD8 when they migrate to specific sites and mature. An injury model inducing focal cerebral ischemia in rats led to an influx of CD8+ macrophages/microglia early after trauma. The number of CD8+ cells peaked at day 6 before returning to normal (41). The decrease in CD8+ cells contrasted with the increase of CD4+ macrophages absent on day 3 after ischemia was induced. The appearance of CD8+ cells is specific for the type of injury provoked in the brain since, induction of optic nerve damage did not result in an accumulation of CD8+ cells at the injury site (41).

Interesting results were found in a similar injury model in mice (136). Ischemia was induced in mouse brain after adaptation of the technique from the rat model. In contrast to the rat, expression of both CD4 and CD8 antigens was low to absent in the mouse model, although the infiltration of the injury site by macrophages was observed. This suggests that under similar conditions such as ischemia, rat and mouse immune response involves the same cells but these cells differ in their expression of markers and, thus, maybe in their mechanisms of action. This is rather surprising since mouse and rat species are often compared to one another for expression of similar markers or mechanisms of immune responses. Interestingly, both rats and mice express CD8α on DC.

Differential marker expression between rat and mouse

Rats and mice can differ significantly in their expression of surface markers. Another example of differential expression of CD8 on rat and mouse cells is NK cells. Rat NK cells express a homodimer of CD8α (45,46), whereas mouse NK cells do not (47,48). The pattern of Thy-1 expression, which was originally described as a marker for

intra-thymic development, also differs between rat and mouse (137). In mouse, Thy-1 can be used as a marker to identify peripheral T lymphocytes. In rat, Thy-1 is mainly expressed on cells in brain, bone marrow and thymus, but not on peripheral T lymphocytes. An example involving differential marker expression on macrophages, which might be more relevant to the present study, is the expression of CD4 on macrophages. Rat and human macrophages express CD4, whereas mouse macrophages do not (138,139).

Conclusions

Thus, arguments can be found to support our hypothesis that mouse macrophages express CD8. Research using rat or mouse systems often outline similarities in immune responses between these two species. However, there are a number of differences between rat and mouse, specifically in expression of CD8 on different cell types such as NK cells. Our findings clearly identified CD3 negative cells within mouse lung that express CD8. The cell type bearing CD8 expression remains to be identified, although a population of AM is a likely candidate.

II. FUTURE DIRECTIONS

A. To expand the findings provided by single-cell RT-PCR

The total number of cells studied using the single-cell RT-PCR technique is 16. This number must be increased to verify the reproducibility of our results. It will also be important to sequence the products obtained by PCR and compare them to T cell CD8α sequence. Moreover, future studies need to include the use of markers other than CD8 and CD3 to clearly identify the cells that are $CD8^+$ but $CD3^-$. The proposed markers must target cell types that are the most likely to be found in BAL fluid. F4/80 or CD68 on macrophages, CD56 for NK cells, CD19 for B cells, elastase for neutrophils and CD11c or CD209 for DC would be appropriate markers for the identification of the $CD8^+$ cells found in BAL from mouse lungs. Optimization of the technique will be required so that more studies can be performed on one cDNA sample.

B. To find an Ab that recognizes CD8 on mouse AM by flow cytometry

The first step could be to test D9 Ab on mouse splenic cells and AM by flow cytometry. D9 Ab was raised against a CD8α peptide including the Ig-like region and part of the hinge region and has been characterized for binding mouse, rat and human CD8α. As G28 (Ig-like domain) in the rat, LT8 could not detect CD8α on human macrophages, while D9 did as OX8 (hinge region) did in the rat. If we postulate that human CD8α on macrophages is similar to CD8α on rat macrophages, the hypothesis could be that the epitope recognized by D9 Ab is outside the Ig-like region proposed

to be different in rat CD8 macrophages. If it is the case, D9 Ab could be a valuable reagent to study CD8 on mouse AM assuming that CD8 structure on mouse macrophages is similar to the novel CD8 on rat and possibly human macrophages.

Future production of additional monoclonal Ab to CD8α hinge region could be considered if the study using D9 Ab fails. Moreover, treatment of cells with reagents such as neuraminidase could answer some questions about the glycosylation state of CD8 on AM. As proposed by rat studies, it is possible that CD8α detection on AM failed because of increased CD8α glycosylation on AM.

An Ab recognizing CD8α on mouse AM would be essential to further study CD8 structure and purify CD8 protein from AM for sequencing.

C. To extend our studies to other cell types in the mouse

CD8 expression has been observed in a mouse MC line. These results need to be verified using freshly isolated MC rather than cell lines which are often not representative of *in vivo* cells. The isolation of relatively pure cell populations from the lungs, or peritoneal cavity could be done using magnetic beads attached to Ab binding specific cells. The labeled cells can then be sorted using an autoMACS cell sorter (Miltenyi Biotec) leading to marked enrichment of a particular cell population.

D. To pursue the study of CD8 in C57Bl/6 mice

The relevance of our observations needs to be established using other strains of mice. C57Bl/6 BAL samples have been studied for CD8 expression and it was found that

one sample expressed CD8 but not CD3, suggesting that CD8 is expressed by cells other than T cells. These studies need to be pursued to identify which cells are expressing CD8 in C57Bl/6 BAL samples. Moreover, characterization of CD8 expression on AM from C57Bl/6 mice will be essential if CD8 functions studies are to be conducted using CD8 knockout mice which have a C57Bl/6 background. Experiments using CD8 deficient mice would be valuable to differentiate between adhesion and signaling functions of CD8 on macrophages.

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